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# CRISPR/CAS9-BASED ENGINEERING OF *ASPERGILLUS NIGER* FOR THE IMPROVED FERMENTATION OF PECTIN-RICH MATERIALS

Master of Science Thesis

Examiners: Docent Peter Richard, Assistant professor Ville Santala and D.Sc. Joosu Kuivanen
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# ABSTRACT

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Millions of tons of pectin-rich materials, such as citrus peel, are annually discarded worldwide. Filamentous fungus *Aspergillus niger* naturally degrades pectin-rich materials. Consequently, *A. niger* has been previously engineered to convert the main component of pectin, D-galacturonic acid, into mucic acid, a value-added chemical. However, the productivity of mucic acid from pectin-rich materials in *A. niger* is not yet high enough for the production to be cost-effective in industrial scale. Strains can be modified in order to increase the yield, the titer and the productivity of specific compounds through metabolic engineering that uses genome editing tools, such as the highly efficient CRISPR/Cas9 system. Unfortunately, the CRISPR/Cas9 system has only been applied to filamentous fungi for a couple of years and lacks a standardized method for *A.niger*.

The aim of this study was to improve the production of mucic acid in *A. niger* from pectin-rich materials through metabolic engineering. As a consequence, an efficient, fast, and simple CRISPR/Cas9 method was tested, downscaled, and optimized for *A. niger* using homologous recombination and the transformation of a *in vitro* synthesized Cas9 and guide RNA as a single ribonucleoprotein. The developed CRISPR/Cas9 method performed with 100 % replacement efficacy when a single gene was replaced with heterologous genes. Method was studied for multiplex genome editing and succeeded in replacing 2 or 3 target genes by heterologous genes. This was notably the first time, RNP-mix-based method was used and multiple gene replacement were performed simultaneously in *A. niger*. In addition, the method is compatible for 96-microwell format in high-throughput workflows for example in robotics.

Multiplex gene editing was performed in *A. niger* in order to improve the production of mucic acid: a gene encoding for pectin-catabolism-repressing transcription repressor protein, *gaaX,* was replaced by an enzyme converting galacturonic acid into mucic acid, while this replacement was combined with the patented pathway. In the 5-days-lasting cultivations with 20 g/l citrus pectin, the new strain produced 12.05 g/l of mucic acid while the old strain produced only 7.31 g/l of mucic.

# TIIVISTELMÄ

**VEERA KORJA**: *Aspergillus niger* -homeen muokkaus CRISPR/Cas9-menetelmällä pektiinipitoisten materiaalien fermentoinnin parantamiseksi
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Avainsanat: CRISPR/Cas9, musiinihappo, galaktaarihappo, pektiini, Galakturonihappo, *Aspergillus niger*

Maailmanlaajuisesti miljoonia tonneja pektiinipitoisia materiaaleja, kuten sitrushedelmien kuorta, heitetään vuosittain pois. *Aspergillus niger* -home hajottaa luonnostaan pektiinipitoisia materiaaleja. Tämän seurauksena *A. niger* on aiemmin geenimuokattu muuntamaan pektiinin pääkomponentti, D-galakturonihappo, musiinihapoksi, joka on arvokas yhdiste. Musiinihapon tuottavuus pektiinipitoisista materiaaleista ei ole kuitenkaan vielä tarpeeksi korkea taloudelliseen teolliseen mittakaavan tuotantoon. Mikrobikantojen metaboliaa voidaan muokata, jotta halutun yhdisteen saanto, pitoisuus ja tuottavuus nousevat. Metabolian muokkauksessa käytetään genomimuokkaustyökaluja, kuten CRISPR/Cas9-systeemiä. Valitettavasti CRISPR/Cas9-systeemiä ei ole käytetty homeisiin kuin muutaman vuoden ajan, ja standardoitu metodi *A. niger* -homeelle puuttuu.

Tämän tutkimuksen tavoitteena oli parantaa metabolian muokkauksella musiinihapon tuottavuutta *A. niger* -homeessa pektiinipitoisista materiaaleista. Tätä varten uusi, tehokas ja yksinkertainen CRISPR/Cas9-menetelmä kehitettiin ja optimoitiin pienempään mittakaavaan. Menetelmässä *A. niger* transformoitiin ribonukleoproteiinilla (RNP), joka sisälsi *in vitro* valmistetut Cas9- ja gRNA-molekyylit. Kehitetty CRISPR/Cas- menetelmä korvasi yksittäisen geenin heterologisilla geeneillä 100 % tehokkuudella. Menetelmä toimi samanaikaisesti usean geenin poistossa, jossa onnistuttiin samaan aikaan korvaamaan 2 tai 3 geeniä heterologisilla geeneillä. Tämä oli tiettävästi ensimmäinen kerta *A. niger* -homeelle, kun käytettiin RNP-kompleksiin pohjautuvaa menetelmää ja useampia geenejä korvattiin samaan aikaan. Lisäksi, menetelmä soveltuu käytettäväksi 96-kuoppalevyformaattiin esimerkiksi pipetointirobotissa.

CRISPR/Cas9-menetelmällä poistettiin geenejä *A. niger* -homeesta, jotta musiinihapon tuottavuus nousisi: pektiinin hajotusta estävän transkriptiorepressorin geeni, *gaaX,* korvattiin entsyymillä, joka muuntaa galakturonihappoa musiinihapoksi. Samaan aikaan geeninvaihto yhdistettiin aikaisemmin patentoituun metaboliareittiin. Viiden päivän jälkeen 20 g/l pektiiniä sisältävissä kasvatuksissa, uusi *A. niger* kanta oli tuottanut 12,05 g/l musiinihappoa, kun vanha kanta puolestaan oli tuottanut vain 7,31 g/l musiinihappoa.

# PREFACE

This master's thesis was conducted in VTT Technical Research Centre of Finland in the Production Host Engineering team from May 2017 to April 2018.

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Veera Korja

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# LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| Cas | CRIPSR associated proteins |
| Cas9 | CRISPR associated protein-9 nuclease |
| CAZY | carbohydrate-active enzymes |
| CRISPR | clustered regularly interspaced palindromic repeats |
| CRISPR/Cas | immune system mechanism used as genome editing tool |
| CRISPR/Cas9 | most commonly used CRISPR/Cas genome editing tool |
| dDNA | donor DNA |
| FDCA | 2,5,- furandicarboxylic acid |
| GA | D-Galacturonic acid |
| GaaA | galacturonic acid reductase |
| GaaB | L-galactonate hydratase |
| GaaC | 3-deoxy-L-threo-hex-2-ulosonate-aldolase |
| GaaD | glyceraldehyde reaductase |
| GaaR | transcription activator related to pectin catabolism |
| GaaX | transcription repressor related to pectin catabolism |
| HG | homogalacturonan |
| MA | mucic acid, *meso*-galactaric acid |
| MoClo | modular cloning |
| PCR | polymerase chain reaction |
| PEF | polyethylene furonate |
| PET | polyethylene |
| RG-I | rhamnogalacturonan I |
| RG-II | rhamnogalacturonan II |
| RNP | ribonucleoprotein containing Cas9 protein and gRNA |
| rpm | rounds per minute |
| RT | room temperature |
| *UDH* | uronate dehydrogenase |
| XG | xylogalacturonan |
| *39114* | gene coding for enzyme related to mucic acid catabolism |
| WT | wild type |

# 1. INTRODUCTION

The amount of genomic data has risen exponentially during the last ten years. This development has led together with new synthetic biology tools, such as CRISPR/Cas9, to the increase in genetic engineering of various microorganisms for industrial applications (Keasling 2003; Deng *et al.* 2017). The potential of microorganisms for the production of renewable chemicals can be harnessed through metabolic engineering, which uses the tools of genome editing. Metabolic engineering comprise the editing of genes and pathways and their regulation in order to produce valuable products. Either organisms' natural pathways of production are enhanced, or heterologous genes or entire pathways are removed into platform organisms. In addition, genes can be deleted, replaced or mutated in host microorganism for example to block competitive pathways or to modify enzymes. Last, the expression of genes can be altered through for example different promoters and transcription factors. (Nielsen and Keasling 2016; Wakai *et al.* 2017) While often the cell's productivity and stability decreases when cells' tightly evolved and regulated metabolism is interfered, metabolic engineering can enable the increase in yields, titres and productivity of chemical compounds in fermentations of microorganisms (Nielsen and Keasling 2016).

While the circular economy is the hot topic of the day, microorganisms have also been engineered to convert inexpensive biomass residues and side streams into value-added chemicals. One attractive waste stream is pectin-rich materials, such as citrus peel and sugar beet pulp. Millions of tons of pectin-rich processing waste are annually discarded around the world (Doran-Peterson *et al.* 2008; Mamma *et al.* 2008; Richard and Hilditch 2009). Some of these waste streams are used as animal feed but the required treatment by drying consumes a lot of energy. Pectin can be extracted from pectin-rich materials and is further used as gelling agent, but the food industry markets are too small to cover the pectin supply resulting from the waste streams. Therefore, large quantities of inexpensive pectin-rich materials remain to be exploited. (Richard and Hilditch 2009) One option to use pectin-rich materials is the conversion to mucic acid (MA), a six-carbon dicarboxylic acid (Mojzita *et al.* 2010).

MA, also known as *meso*-galactaric acid, is a chemical compound that can be used in skincare products and as a precursor for several polymer precursors, such as adipic acid and 2,5-furandicarboxylic acid (FDCA). Adipic acid is a widely used petroleum based chemical which is predominantly used as a building block in nylon.(Lewkowski 2001) As for FCDA, it can be used as a precursor for the renewable plastic polyethylene furanoate (PEF). PEF has the potential to replace polyethylene terephthalate (PET), used in petroleum based plastics in the market. (de Jong *et al.* 2012; Li *et al.* 2014)

Although, MA can be produced through several chemical steps, an alternative option for the production is a single biological process from D-galacturonic acid (GA) that is abundantly available as a main component in pectin. The filamentous fungus *Aspergillus niger* is one of the organisms that has been previously engineered to produce MA from pectin-rich materials by disrupting its native catabolic pathways for GA and MA. In order to gain MA producing strain, two genes D-galacturonic acid consuming *gaaA* and MA consuming *39114* have been deleted and another gene introduced: bacterial urinate dehydrogenase, *UDH*, converting GA to MA. Although, the obtained MA yield was at high level, the productivity is not yet high enough to be cost effective for industrial use. (Kuivanen *et al.* 2016)

*A. niger* secretes naturally pectin degrading enzymes (i.e. pectinases) at high level and is therefore an excellent platform organism to convert pectin-rich biomass to different products (Martens-Uzunova and Schaap 2009; Kuivanen *et al.* 2016). The pectin degrading enzymatic network in *A. niger* is very complex and tightly regulated (Kowalczyk *et al.* 2017). In 2017, Niu et al. reported that a repressor protein GaaX affects significantly the production of pectinases and pectin related proteins in this network by restraining the activity of the activator protein GaaR. Consequently, the deletion of *gaaX* results in continuous induction of pectinase and transporter protein production (Niu *et al.* 2017; Alazi *et al.* 2018).

This study aims to improve the MA production from pectin-rich materials in *A. niger*. The first aim was to delete *gaaX* gene in the MA producing *A. niger* platform strain of Δ*gaaA* Δ*39114 UDH* as presented in Figure 1.1 . However, tools for genetic engineering in filamentous fungi are still relatively underdeveloped and especially targeted genome editing has been challenging due to inefficient homologous recombination. Recently, CRISPR/Cas9 tools have facilitated genome editing also in filamentous fungi. (Sander and Joung 2014; Deng *et al.* 2017). However, these CRISPR/Cas9 systems include usually laborious cloning and *in vivo* expression of Cas9 and gRNA in the cells. Consequently, the second aim of this study was to develop a simple and efficient CRISPR/Cas9 genome editing method for *A. niger* capable of deleting multiple genes simultaneously.

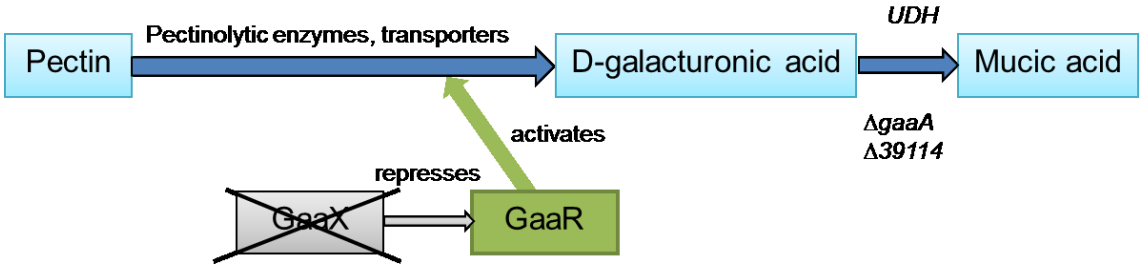



***Figure 1.1*** *The deletion of gaaX should result in improved production of MA from pectin in A. niger fermentation*

The second chapter presents the theory and motives behind this study in detail. Chapter 3 reports the materials and methods including cloning, CRISPR/Cas9 and MA production in *A. niger*. Results are shown in chapter 4 and reflected in chapter 5 before the conclusion in final chapter 6.

# 2. THEORETICAL BACKGROUND

## 2.1 Production of mucic acid from pectin in *A. niger*

Fermentation of industrially relevant filamentous fungus *A. niger* can be used for MA production from available and cheap pectin-rich materials, such as citrus pectin and sugar beet pulp. Higher MA productivity may be achieved through metabolic engineering of *A. niger*. First step includes analysing the pathways and gene regulation in pectin, GA and MA metabolism in *A. niger*.

### 2.1.1 *Aspergillus niger*

*A. niger* is a haploid, asexual filamentous fungus that belongs to the genus of *Aspergilli* (Mable and Otto 1998). It produces black uninucleate spores, conidia, that are spread through air (NGA *et al.* 1975). *A. niger* is a saprotrophic mould and thus lives by aerobic degradation of dead plant material by secreting a wide variety of enzymes for the degradation of plant biomass. Many of *A. niger* products are officially considered safe with GRAS-status (generally recognized as safe). *A. niger* is highly tolerant of different conditions and substrates, and therefore appears almost everywhere in nature. It grows in different of temperatures (6 ºC – 47 ºC) and pH (1.4 – 9.8), but it prefers the conditions of 35 ºC -37 ºC and acidic pH. (Schuster *et al.* 2002; Knuf and Nielsen 2012)

*A. niger* is an industrially important microorganism. It naturally produces oxalic acid and various different enzymes for biomass degradation depending on the substrate. Wild type *A. niger* ATCC1015 has been used for citric acid production in specific culture conditions since 1919. Nowadays more than 1.6. million tons of citric acid is produced by fermentation of *A. niger*. Citric acid is applied mainly in food industry as an acidifying agent for example in lemonades, but has also various other applications in pharmacology, cosmetics, detergent and chemical industry. (Roukas 2000; Schuster *et al.* 2002) Another organic acid produced by *A. niger* fermentation is gluconic acid. More than 60 tons of it is annually used around the world in food and pharmacological industry for acidification, flavour enhancement and bakery leavening. (Roukas 2000; Ramachandran *et al.* 2006)

Since *Aspergillus* also produce a wide variety of enzymes, it has been widely used for enzyme production worldwide as well as in Finland (Aittomäki *et al.* 2002, pp. 113). One of the enzyme groups, that *A. niger* produces and secretes, is pectin degrading enzymes, pectinases. Different pectinases are used for the food and feed processing, for example in juice clarification. (Kowalczyk *et al.* 2017; Niu *et al.* 2017) Another important fungus in *Aspergilli* is *Aspergillus oryzae.* It has been used for food fermentation in Japan for centuries and it produces also a wide variety of enzymes for industrial applications, like *A. niger* (Schuster *et al.* 2002; Knuf and Nielsen 2012).

### 2.1.2 Pectin and pectin-rich materials

Pectin is common name for a diverse group of natural heteropolymers that contain mainly D-galacturonic acid, GA. (Martens-Uzunova and Schaap 2009) GA is an aldose sugar with acid group. In other words, GA is an uronic acid. In pectin, about 80% of the galacturonic acid's carboxyl groups are methylated. Pectin forms out of four different polysaccharides: homogalacturonan (HG), rhamnogalacturonan I (RG-I), rhamnogalacturonan II (RG-II) and xylogalacturonan (XGA) which form out of several different molecules as seen in Figure 2.1 and 2.2. (Richard and Hilditch 2009) Pectin has two even bigger structural types. First structure has HG and RG-I varying in the backbone and side chains of neutral sugars, while the second has RG-I as backbone and sidechains of HG and sugars. (Martens-Uzunova and Schaap 2009).

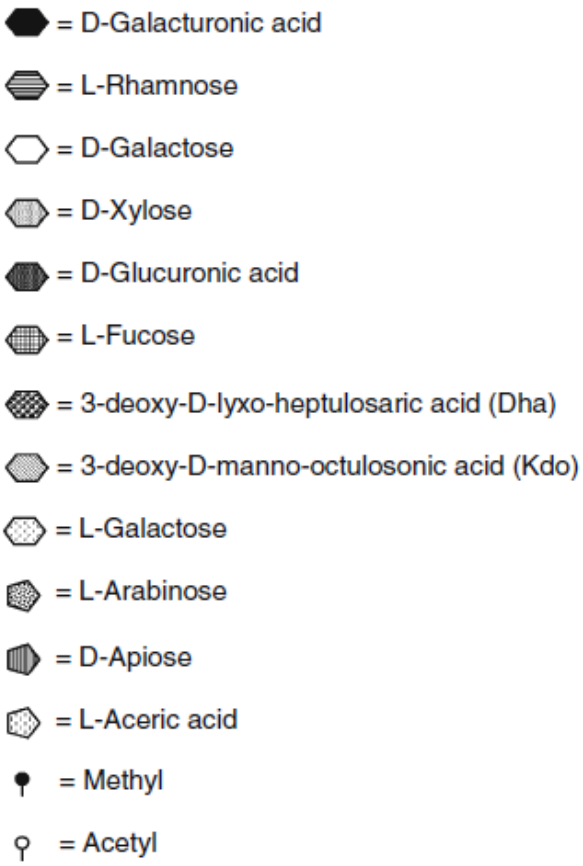



***Figure 2.1*** *Components in pectin (Richard and Hilditch 2009, pp 598).*

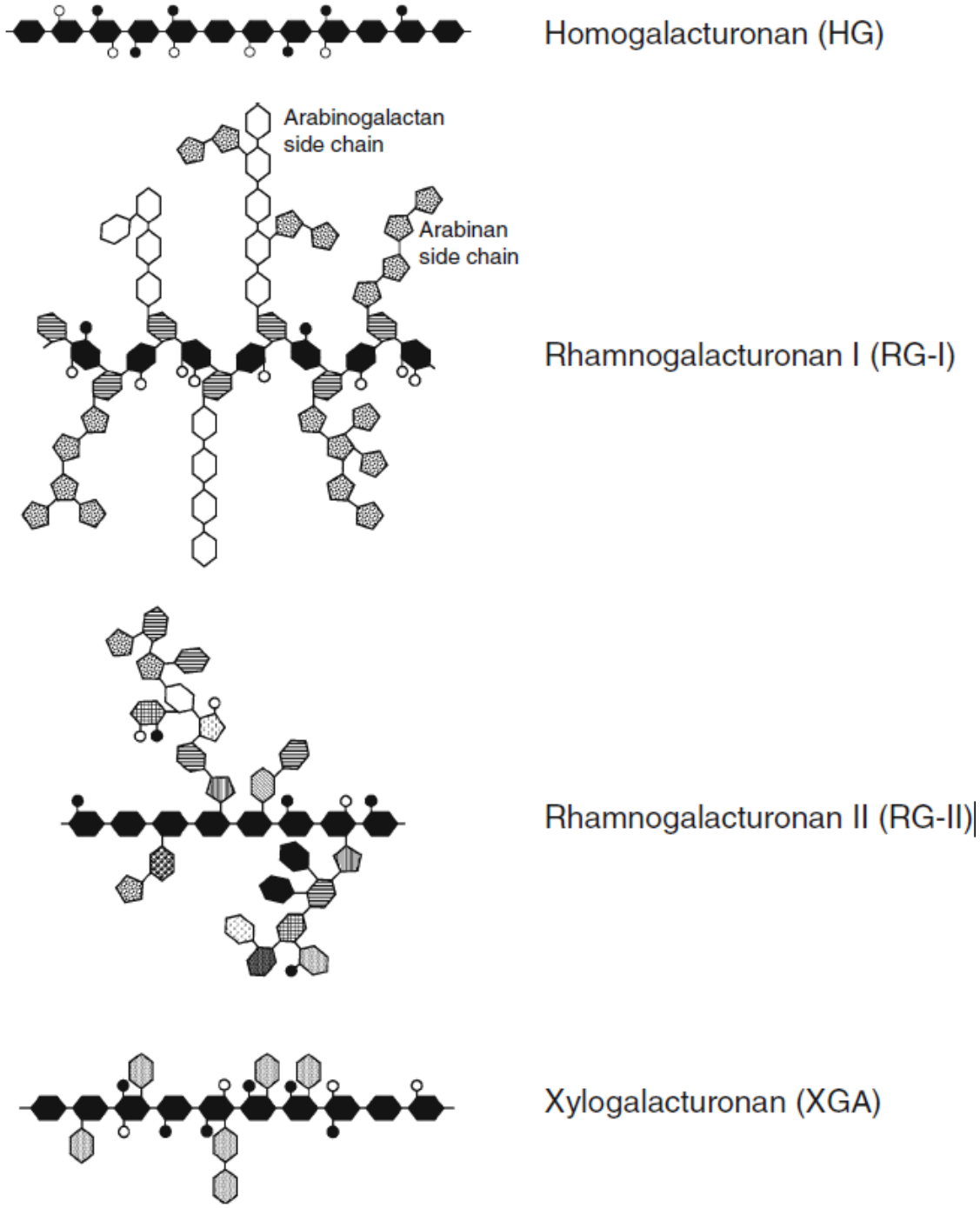



***Figure 2.2*** *The four forms of polysaccharides in pectin: homogalacturonan, rhamnogalacturonan I, rhamnogalacturonan II and xylogalacturonan (Richard and Hilditch 2009, pp. 598).*

Homogalacturonan is the simplest form of pectin as it contains only a linear polymer where GA residues are linked through hydroxyl groups in C1 and C4. GA residues in HG can also contain methyl and acetyl sidechains at their carboxylic groups. XGA differs from HG by having xylan or β-D-xylose residues linked to C3 of the GA residues in the backbone. Possibly the most common form of pectin, RG-I contains a backbone of disaccharides with two alternating residues: α-(1,2)-linked rhamnosyl and α-(1,4)-linked GA

residues. The linear or branched side chains situated in O-4 of rhamnosyl vary between one neutral sugar and complex polymer. These sugars and polymers are formed for example from galactan, arabinan or arabinogalactan. Perhaps the most complex of them all, RG-II contains rare sugars as sidechains and around nine α-(1,4)-linked GA residues as a backbone. (Martens-Uzunova and Schaap 2009; Richard and Hilditch 2009) The chemical composition of pectin varies between different plants. For example, rhamnogalacturonan side chains and the length of HG are different between citrus plants. The structure of pectin can also vary within the same species between tissues and stages of maturation. (Kaya *et al.* 2014)

Pectin is found in the middle lamella of the primary cell wall in dicotyledonous plants and forms one third of these cell walls. Pectin is important for cell growth, mechanical strength and defence mechanisms. (Kaya *et al.* 2014). Pectin-rich residues form for example in apple and citrus juice production and sugar beet processing. Dried citrus peel contains 20 % pectin whereas dried sugar beet 24%. (Doran-Peterson *et al.* 2008)

Due to pectin's chemical properties, pectin forms a gel in water if it contains either $Mg^{2+}$ or $Ca^{2+}$ bivalent cations or sugars as high concentration in acidic pH. Food industry exploits citrus pectin's gelling property, and uses pectin as a gelling agent in jams, marmalades and jellies. Other industries as well, such as pharmaceutical, has taken advantage of these gelling, thickening and emulsifying properties of citrus pectin. However, sugar beet pectin contains so much acetyl groups that it does not have gelling characteristics. Although, pectin is used in the food industry, it does not provide any nutrional advantage due to its complex chemical structure. Only ruminants can digest pectin further, and therefore, pectin-rich material is sold as cattle feed. (Richard and Hilditch 2009)

Citrus peel and sugar beet pulp are washed, dried, and pelletized to secure its preservation through the transport and storage. Pelletized product is commonly used as cattle feed. (Richard and Hilditch 2009; Kaya *et al.* 2014) However, this treatment process is highly energy consuming. For example the drying and pelletizing step consumes 30-40% of the required energy of the whole cattle feed manufacturing process from sugar beet pulp(Doran *et al.* 2000). This is why citrus peel or sugar beet pulp processing has to be done in large scale to make profit. In addition to usage as cattle feed, dried peel, or pulp can be used for pure pectin extraction through acid hydrolysis in high temperature. Pure pectin is used for example in food industry (Richard and Hilditch 2009).

Millions of tons of citrus peel and sugar beet pulp are formed annually. While food industry does not require such amounts of pectin-rich materials to make gelling agents and drying process of pectin-rich materials is not cost-effective, large portion of citrus peel and sugar beet pulp is discarded to landfills, especially in developing countries. This is why it is in high interest to develop new ways to use pectin-rich materials. (Richard and Hilditch 2009)

One possibility to use available and cheap pectin-rich materials is the fermentation of them into value-added chemicals. Since some microorganisms naturally degrade pectin and its main component GA, they contain enzymes that catabolize pectin into other molecules. These pathways can be combined with heterologous genes so that valuable chemicals are produced, for example ethanol in *Escherichia coli* (Doran *et al.* 2000)and L-ascorbic acid and L-galactonic acid in *A. niger* (Kuivanen *et al.* 2014, 2015) or MA in *A. niger* or *Trichoderma reesei* (Mojzita *et al.* 2010; Kuivanen *et al.* 2016).

### 2.1.3 Degradation of pectin and galacturonic acid in *A. niger*

As pectin forms a big portion of some plants' cell walls, it is an important source of carbon for different organisms degrading plants, such as bacteria and fungi. Consequently, many fungi secrete enzymes that catabolize pectin. *A. niger* produces various extracellular enzymes that take part in pectin degradation. As pectin structure is complex and varies between plant species, the pectin degradation requires several different enzymatic activities. (Martens-Uzunova and Schaap 2009)

Numerous pectinolytic enzymes have been characterized since 1990s. Pectin degrading enzymes are divided roughly into two categories: Pectinases catabolize the backbone of the pectin while accessory enzymes catabolize the various sidechains. Pectinases include various different hydrolases, lyases and methyl and acetyl esterases, which function either inside or outside the cells. Most of the pectinases belong to carbohydrate active enzyme (CAZY) families GH28, PL1, PL4, CE1, CE8 and CE12. (Martens-Uzunova and Schaap 2009)

In 2009, Martens-Uzunova and Schaap first assessed all pectinolytic enzymes by studying transcriptional levels of different genes in various media including pectin, galacturonic acid, xylose and rhamnose. All together 46 genes have been identified to encode a protein related to pectin degradation. For example only HG is degraded by 23 different pectinases including endo- and exo-polygalacturonases, pectin and pectate lyases and pectin methylesterases. Exo-polygacturanases and endo-xylogalacturonan hydrolases degrade XG. RG is catabolized by endo- and exo- rhamnogalacturonases, rhamnogalacturonan lyases, rhamnogalacturonan acetyl estarases and unsaturated rhamnogalacturonyl hydrolases. Different side chains in rhamnogalacturonan are degraded for example by different arabinofuronosidases, galactosidases and endo-arabinases and –galactanases, Altogether, different pectinases and accessory enzymes catabolize pectin into smaller molecules from different positions. These enzymes can act constitutively or inductively under different conditions. (Martens-Uzunova and Schaap 2009)

Since GA is the main constituent in pectin, it is only logical that *A. niger* can use it as carbon source after pectin has been degraded into monomers. In original fungal pathway in *A. niger*, *gaaA, gaaB, gaaC* and *gaaD* encoded enzymes convert reaction by reaction

GA into glycerol and *pyrG*uvate. This pathway is presented in Figure 2.3. First, D-galacturonate reductase, GaaA, reduces GA into L-galactonic acid. Second, GaaB converts L-galactonic acid into 3-deoxy-L-threo-hex-2-ulosonate (2-keto-3-deoxy-L-galactonic acid). GaaC, an aldolase, cleaves this last molecule in two: *pyrG*uvate and L-glyceraldehyde. GaaD, glyceraldehyde reductase, finally reduces the L-glyceraldehyde product into glycerol. (Martens-uzunova and Schaap 2008; Richard and Hilditch 2009)

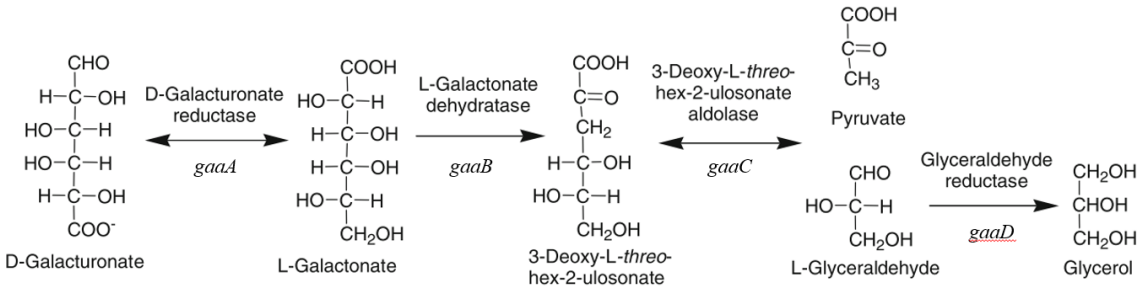



***Figure 2.3*** *Natural D-galacturonic acid catabolism in A. niger, modified from (Richard and Hilditch 2009, pp 601).*

Deletion of *gaaA* and *gaaD* results in *A. niger* that has reduced growth and sporulation with GA and polygalacturonic acid as carbon sources. The growth collapses when *gaaB* or *gaaC* is deleted. As a consequence, *A. niger* has some still unknown redundant enzymes to replace the functions of GaaA and GaaD but not the functions of GaaB and GaaC. (Alazi *et al.* 2017)

### 2.1.4 Regulation of pectin degrading pathways in *A. niger*

Transcription factors, TFs, regulate gene transcription by binding to promoter areas of genes or to other TFs or inducer molecules. Finally some of the regulatory proteins attach to specific motifs upstream from the start codons. Transcription of proteins is controlled by TFs so that right proteins are produced at right moments. Many TFs activate or repress gene transcription after activation by specific inducers, often related to enzymes' substrates. *A. niger* controls the production of plant degrading enzymes and has many TFs. Especially sugar-dependent TFs exist in *A. niger*. Constitutively expressed enzymes release inducers, sugar monomers or other molecules, in under certain conditions. These enzymes ensure that change in conditions is noticed in metabolic level when inducer molecules' concentrations increase. (Alazi *et al.* 2017; Kowalczyk *et al.* 2017)

*A. niger* regulates the pectin degradation with several TFs and inducer molecules. Three most important transcription activators are GA-responsive GaaR, L-rhamnose-responsive RhaR and L-arabinose-responsive AraR. They affect the production of pectinolytic endo- and extracellular enzymes as well as sugar transporters. GaaR is a TF that activates mostly the production of proteins that degrade HG, convey D-galacturonic acid in the cell and catabolize D-galacturonic acid. Second TF is RhaR. It mostly controls proteins that are

involved in RG-I decomposition where RhaR controls the release, uptake and metabolism of L-rhamnose. Third relevant TF in pectin degradation is AraR that has control over RG-I sidechain catabolism where L-arabinose is released. It also activates the uptake and metabolism of L-arabinose. (Kowalczyk *et al.* 2017)

As many TFs regulate the degradation of pectin, *Kowalczyk et al* studied their combinatorial effect in 2017. Transcription profiles of triple, double and single mutant strains were analyzed in different cultivation media where alternative mutant strains had *gaaR, rhaR* and *araR* deleted. As a result, GaaR had most effect in pectin degradation since, in addition to having control over tens of enzymes and sugar transporters related to HG degradation, it regulates genes that are also under control of RhaR and AraR. This means double or even triple regulation over some genes related to pectin degradation. In this trio, RhaR had the least effect on expression of pectinolytic genes when *A. niger* was grown on sugar beet pectin. Moreover, the cultivation time had effect on the regulation of pectin degrading pathways. (Kowalczyk *et al.* 2017)

In addition to three transcription activators, there are two transcription repressors GaaX and CreA related to pectin degradation. CreA is a general carbon catabolite repressor that was reported to repress nine GA-responsive genes. Even more important repressor was shown to be GaaX that is expressed from *gaaX* just next to *gaaR* in *A. niger* genome. The deletion of *gaaX* resulted in upregulation of various GA-responsive, GaaR activated genes. Niu et al proposed panregulon with 53 GaaR-GaaX regulated genes and core regulon of at least 27 genes. This GaaX-GaaR, activator-repressor module was also found to be conserved in various ascomycetes filamentous fungi, such as *A. oryzae*. (Niu *et al.* 2017)

Some pectin related genes are constitutively expressed at low level, for example *pgaA* and *pgaB* that encode endo-polygalacturonases. The activity of these enzymes produce inducers that have effect on TFs. GA-responsive, GaaR regulated genes are induced in the presence of GA resulting for example from the activity of *pgaA* on HG. GaaR was proposed to be activated by GA or its derivate. In 2017, *Alazi et al* suggested the inducer molecule to be 2-keto-3-deoxy-L-galactonate, the product of GaaB and the substrate of GaaC. When *gaaC* was deleted, the inducer molecule accumulated in *A. niger* and induced GA-responsive GaaR-activated genes (Alazi *et al.* 2017).

As pectin is degraded partly by constitutive enzymes, some GA ends up inside *A. niger* and is converted into inducer molecules. As proposed by Alazi *et al.*, when 2-keto-3-deoxy-L-galactonate inducer is present in higher concentrations, it binds to GaaX. Due to this structural change, GaaX releases GaaR. Free GaaR can respectively activate GA-induced genes by binding to GaaR motifs in their promoters. Finally, pectin degrading pathways are upregulated as seen in Figure 2.4 . (Alazi *et al.* 2017; Niu *et al.* 2017)

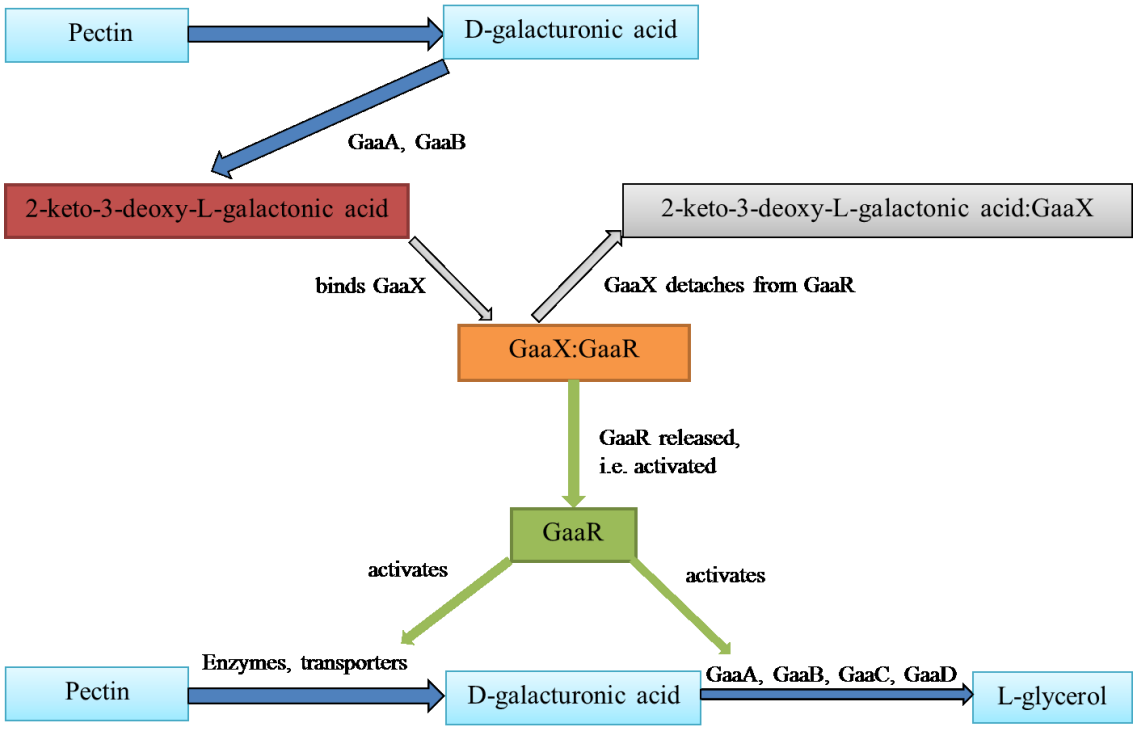



***Figure 2.4*** *The regulation of GA-inductive genes by GaaX repressor and GaaR activator proteins and inducer molecule of 2-keto-3-deoxy-L-galactonic acid: when pectin or GA is present, the concentration of inducer increases, it binds more to GaaX resulting the release and activation of GaaR. Free GaaR activates the GA responsive genes in pectin and GA catabolism.*

Upregulation of GA-responsive genes is desirable when pectinolytic enzymes are being produced or pectin-rich materials are being degraded. According to Niu *et al* and the figure above, when more GaaR is available in free form, GA-responsive genes are upregulated by it (2017). This can be done by deleting *gaaX* or *gaaC*. When *gaaX* is deleted, it does not bind transcription activator GaaR. Alternatively, when *gaaC* is deleted, inducer molecule, substrate of GaaC, accumulates inside the cell, binds GaaX while GaaR is released. When active GaaR is available continuously, it also activates the genes under non-inducing conditions. Consequently, it is most likely that *A. niger* degrades pectin straight from the beginning of cultivations of pectin-rich materials. Δ*gaaX* upregulates the expression of 37 genes: 25 pectinolytic enzymes, 7 sugar transporters and 5 unknown proteins. The most upregulated genes were *gaaA, gaaB, gaaC* and *gaaD*, but also other genes such as GA transporter *gatA* and exo-polygalacturonase *pgaX*. (Niu *et al.* 2017) During this study, a third option was published: the expression of *gaaR* under constitutive promoter. Alazi *et al* concluded that constitutive expression of *gaaR* resulted in higher production of pectinases than deletion of *gaaX* (2018). They also noticed that even under overexpression of *gaaR* some of the exo-polygacturonases were under negative control of CreA.

### 2.1.5 Mucic acid production in filamentous fungi

Mucic acid can be produced with chemical oxidation from D-galacturonic acid but more sustainable option would be to produce it through fermentation of microorganisms. While GA is the main component of pectin and *A. niger* produces pectinases naturally, it is especially an attractive platform for MA production. Unfortunately, *A. niger* also naturally converts GA through a conserved D-galacturonic acid pathway resulting in glycerol. After characterization of bacterial uronate dehydrogenase, an enzyme that converts GA into MA, metabolic engineering of filamentous fungi seemed more possible (Kuivanen *et al.* 2016)

In 2009, *Mojzita et al*, engineered *A. niger* as well *Hypocrea jecorina* to produce MA from GA when *UDH* was introduced in them and the natural fungal GA pathway was disrupted by deletion of *gaaA* from *A. niger* and *gar1* from *H. jecorina*. *H. jecorina* performed better since it produced more MA than *A. niger*, that started catabolizing the resulting MA. An anamorph of *H. jecorina* is industrially relevant *T. reesei* which has been used since for the production of MA. (Mojzita *et al.* 2010)

After optimization of cultivation conditions and media, *T. reesei* produces up to 20 g/l MA from GA based medium (Barth and Wiebe 2017). After scaling up and down hydrolyzed pectin cultivations, *T. reesei* produces 11 g/l of MA in 4 ml cultivation, 21 g/l in bioreactor with volume of 10 l and 14 g/l in 250 l bioreactor. However, a downside to *T. reesei* cultivations is that *T. reesei* does not naturally produce pectinases like *A. niger* does. Consequently, the production of MA with *T. reesei* requires a hydrolysis as pretreatment with expensive commercial pectinases. In addition, the rate and titer of production are not yet sufficient for commercial applications. (Paasikallio *et al.* 2017) As a result, *A. niger* still remained as an interesting platform for MA production although it consumed the MA it produced.

In 2016, Kuivanen *et al,* identified the genes responsible for MA consumption in the metabolism of *A. niger*. Especially a protein encoded from the gene *39114* was shown to be responsible for catabolizing MA. This gene was deleted from a strain with the deletion of *gaaA* and expression of *UDH* under constitutive *gpdA* promoter from *A. fumigatus*. New strain Δ*gaaA* Δ*39114 UDH A. niger* produced MA to cultivations with either GA or citrus peel. This new strain hydrolysed pectin with its natural pectinases, imported GA inside the cells and converted it into MA, which accumulated in the cultivations instead of consuming it. This new pathway seen in figure 4 is patented. (Kuivanen *et al.* 2016; Wang 2016)

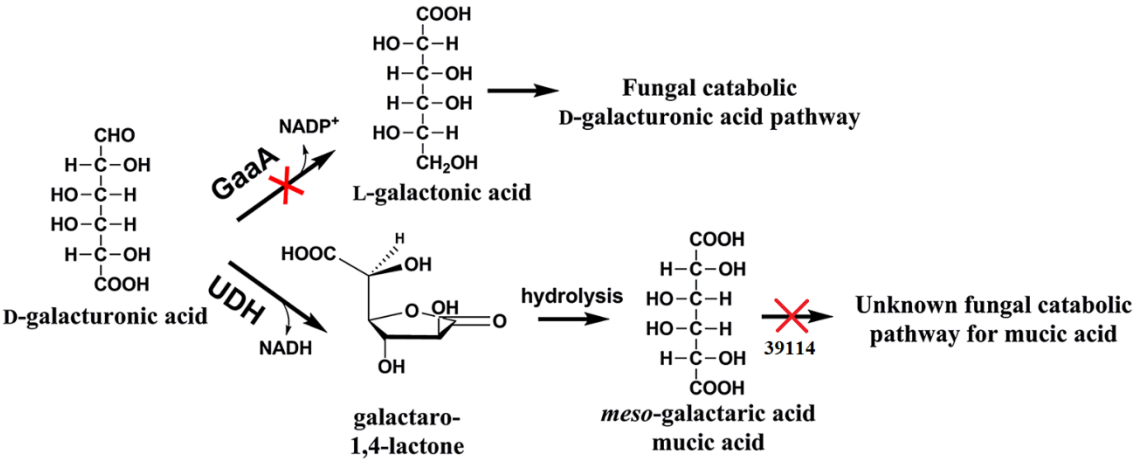



***Figure 2.5*** *Patented MA producing pathway from GA in ΔgaaA Δ39114 UDH strain of A. niger, modified from (Kuivanen et al. 2016, pp. 2).*

Δ*gaaA* Δ*39114 UDH A. niger* produces 4.3 g/l MA from GA with 1:1 ratio in 5 days, but does not use all GA though the process produces energy as NADH as seen in figure above. As a result, *A. niger* needs a co-substrate in addition to GA. As pectin is more complex than GA alone and contains sugars that can be metabolized, Kuivanen *et al* cultivated the strain in citrus peel but it still produced only 3.1 g/l MA from 40 g/l citrus peel waste in 5 days. (Kuivanen *et al.* 2016; Wang 2016) As a result, the rate of MA production from pectin-rich materials with *A. niger* still remains too slow for industrial scale production.

New ideas exist to increase MA productivity from pectin-rich materials. One option is to accelerate the substrate, GA, production from pectin-rich materials. With recent published information about pectin degrading pathways' regulation through GaaX and GaaR (Kowalczyk *et al.* 2017; Niu *et al.* 2017; Alazi *et al.* 2018), one alternative to accelerate GA and further MA production from pectin is to combine the deletion of *gaaX* repressor gene with Δ*gaaA* Δ*39114 UDH* strain. This way *A. niger* would produce faster and more pectinolytic proteins resulting in more GA faster inside the cells to be converted into MA.

### 2.1.6 Structure and applications of mucic acid

MA, also known as *meso*-galactaric acid, is a hexaric acid. This sugar acid with two terminal carboxyl groups is a potential platform chemical for different applications. Already, in 1920s, MA was used as baking acid in self-rising flours and mordant and chrome assistant for dyeing of textiles (Anonymous 1922). Nowadays, it is mostly exploited in skincare products as chelater (Sauermann 2004). Still, the most important way to use MA, is its potential to be converted into different monomers that can be polymerized. (Kuivanen *et al.* 2016)

MA can be chemically converted into adipic acid monomer (Li *et al.* 2014). Adipic acid is processed mainly into Nylon, but it can be used also in various applications from cosmetics to fertilizers and paper. Adipic acid is also used in other polymers, like polyurethane, and in polyvinyl chloride and polyvinyl butyral as a plasticizer. Another option for MA is to convert it to 2,5-furandicarboxylic acid, FDCA, with applications ranging from pharmacology to polymers (Lewkowski 2001a). FDCA can be turned into PEF, polyethylene furonate. PEF can replace fossil-fuel based PET, polyethylene terephthalate, used in plastic bottles. PEF is an interesting biobased plastic, which has even superior characteristics compared to PET. (Li *et al.* 2014) As a conclusion, FDCA can possibly act as a green alternative and renewable chemical to replace fossil fuels in plastic production. (de Jong *et al.* 2012) MA can be converted into other various monomers in addition to adipic acid and FDCA (Taguchi *et al.* 2008).

For larger scale, MA was produced first time in 1920s (Anonymous 1922). It was and is still produced chemically by oxidation of galactose with nitric acid (Acree Salomon Farley 1920; Kiely and Hash 2007). Today the process has high costs and produces hazardous waste. MA can be also converted from galacturonic acid through several chemical steps (Rautiainen *et al.* 2015). Mucic acid and galacturonic acid are presented in figure 3. Chemical processes usually consume a lot of energy and produce toxic wastes. This is why producing MA in a single biological process with microorganisms, like *A. niger, is* an attractive alternative. MA production from cheap and available pectin-rich materials in filamentous fungi was explained in detail in chapter 2.1.5.

## 2.2 Genome editing of filamentous fungi with CRISPR/Cas9

Clustered regularly interspaced short palindromic repeats (CRISPR) are present in different bacterial and archaeal genomes and connected to organism's immune system. CRISPR are part of organism's defence mechanism against viruses and other exterior DNA. CRISPR arrays include CRISPR repeats in regular intervals and variable sequences, called CRISPR spacers, from foreign origins between CRISPR repeats. In this defence mechanism, first small pieces of foreign DNA are cut by CRISPR-associated enzymes (Cas) from foreign DNA and integrated into CRISPR array as spacers in the adaptation phase. Second, in biogenesis phase, CRISPR spacers act as a memory since they are transcribed to CRISPR RNAs (crRNAs), processed into small interfering crRNAs which can recognize foreign DNA through homology. Third, in targeting phase, Cas protein, finds the foreign DNA with the help specific crRNA molecule. Cas9 acts as nuclease and cleaves foreign double strand DNA. As a conclusion, CRISPR/Cas mechanism protects the organism from virus infection and other foreign DNA. (Barrangou and Marraffini 2014)

CRISPR/Cas defence mechanism was first demonstrated as a genetic engineering tool in 2013 and has been used since in genome editing. Meanwhile thousands of articles have been published using CRISPR/Cas technology in different prokaryotes or eukaryotes.

(Hsu *et al.* 2014) Type II CRISPR system based on Cas9 nucleases is the most popular tool among CRISPR systems used for genome editing and other applications. CRISPR/Cas9 technology has gained such an interest because it is simple, versatile and efficient compared to other genetic engineering tools, like transcription activator-like effector nucleases (TALEN) or zinc finger nucleases (ZFN). The design and preparation of CRISPR/Cas modifications include straightforward design of specific gene targeting oligonucleotides. Whereas, the use of ZFN or TALEN, tools found before CRISPR/Cas, require laborious design and preparation of specific gene targeting proteins. (Hsu *et al.* 2014; Sander and Joung 2014; Deng *et al.* 2017)

At the same time as the amount of genomic data is increasing rapidly, the genome editing tools, such as CRISPR/Cas9 are studied as well. While there are more and more interesting research topics, CRISPR/Cas9 methods should be performed with the least laborious cloning effort, with multiple simultaneous deletions, modifications and insertions to the same strain. CRISPR/Cas9 should also be so simple that multiple strains could be handled at the same time. This calls for tryout of new methods, optimization and use of automation in CRISPR/Cas9 methods.

### 2.2.1 CRISPR/Cas9 as a genome editing tool

CRISPR/Cas systems are divided into 3 different categories (I, II and III) and even further into various subtypes. This sorting is made according to genetic, structural and functional differences between the systems, but the emphasis is on different Cas proteins. Still, the most popular and the most characterized CRISPR/Cas system in genome editing and in other applications is CRISPR/Cas9 from *Streptococcus pyogenes* bacterium. Cas9 are signature proteins for type II systems and include conserved RuvC and HNH domains. Each one of these domains are responsible for the cleavage of one strand in the double strand of the DNA. Type II systems are more popular since they function through a single Cas9 and CRISPR RNA (crRNA) and trans-activating RNA (tracrRNA), whereas type I and III systems require difficult synthesis of massive, multimeric complexes including Cas, crRNA and more. (Barrangou and Marraffini 2014)

In CRISPR/Cas9 crRNA and tracrRNA form a duplex. The crRNA contains a 20 bp long sequence homologous to target sequence called protospacer. This is followed by a special protospacer adjacent motif (PAM) sequence, which varies between different Cas9 proteins. PAMs have their complementary sites in the target sequences. In *S. pyogenes*, PAM-site in the target sequence forms out of any nucleotide and two guanines, NGG. After PAM, crRNA has 22 bp sequence that binds to tracrRNA. Although crRNA is the most important in the target recognition, tracrRNA is still a significant factor in regulating the formation of Cas9:crRNA:tracrRNA ribonucleoprotein and final DNA cleavage of the target site. In genome editing through CRISPR/Cas9, the combination of hybridized crRNA and tracrRNA molecules is called a guide RNA (gRNA). Sometimes gRNA is expressed *in vivo* as a single molecule and are called a chimeric single guide RNA

(sgRNA). Another option is to order commercially available specific crRNA and common tracrRNA and form their duplex just as in the natural CRIPSR/Cas9 defence system (Sander and Joung 2014)

Cas9 nuclease and crRNA:tracrRNA duplex bind to each other and form a ribonucleoprotein (RNP) complex. With the help of crRNA binding to the target genomic site by RNA-DNA base pairing, PAM is recognized and RNP-complex binds to target site. Cas9 nuclease generates double-strand break (DSB) 3 – 5 bp upstream from PAM-site as seen in Figure 2.6 . This same system works as a genome editing tool when Cas9 and crRNA:tracrRNA are introduced in the cells. In the nature the Cas9 cleaves foreign DNA but in genome editing it cleaves the target site to be modified. As an automatic response, the cell starts to repair damaged DNA, which is rarely done perfectly nucleotide by nucleotide without donor template. As a result, target gene is often disrupted. (Sander and Joung 2014)

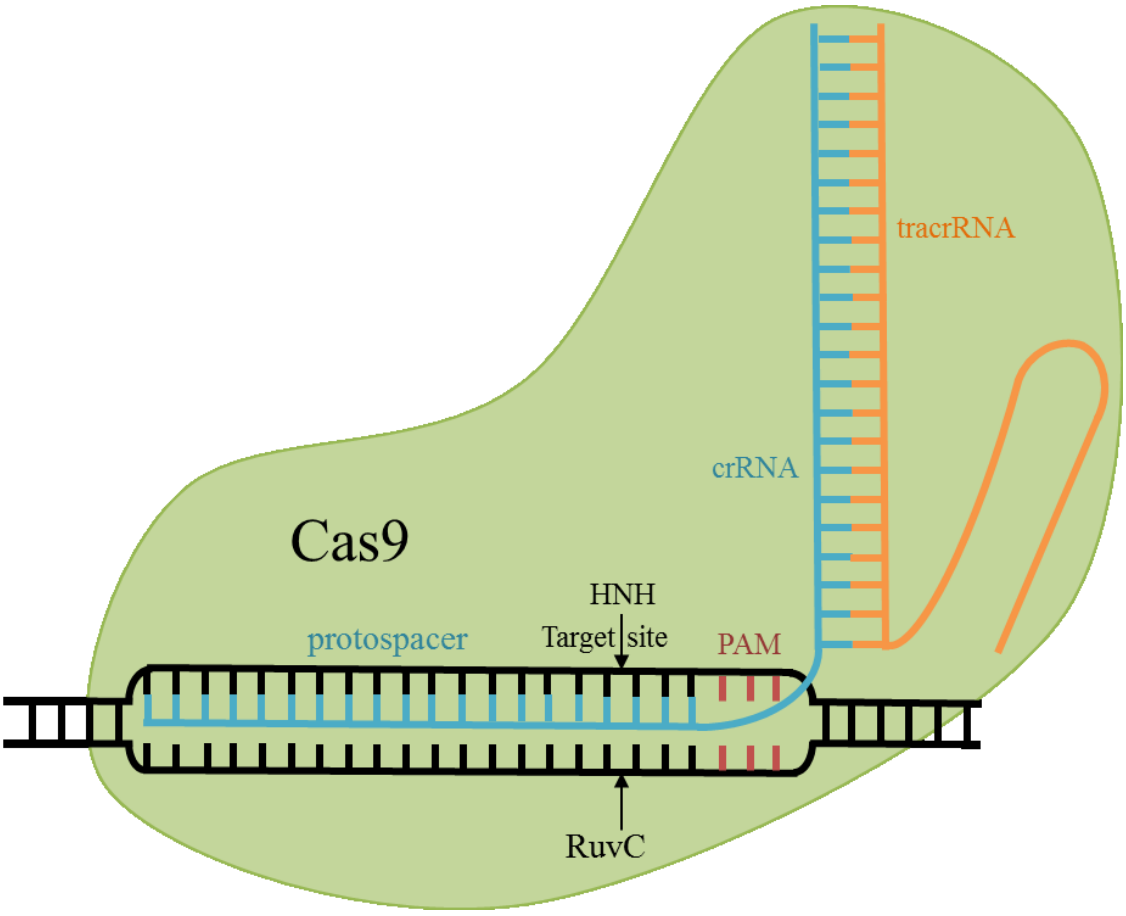



***Figure 2.6*** *Cas9 causes a double strand break to the target site that is 3 bp upstream from PAM. Ribonucleoprotein complex, including CAS9 and crRNA:tracrRNA duplex, recognizes and binds to the target site with PAM and homology between target and protospacer in crRNA. In Cas9, the HNH domain cleaves on strand of DNA as the RucV domain cleaves the other strand.*

Before CRISPR/Cas9 system emerged, the genetic engineering relied mostly on the traditional system of homologous recombination. This system included donor template DNA with homologous flanking sequences to target site. Unfortunately, this system worked with a very low efficiency and needed always a selection marker integrating into the genome, and multiple simultaneous deletions were only dreamed of. Only one published study was found to contain TALENs used for filamentous fungi. CRISPR/Cas9 system has been studied in filamentous fungi only for couple of years. It has been studied only in some species with a few different techniques. Since filamentous fungi can be used in various fields, such as drug discovery, bioenergy and high value chemicals, it is in high current interest to develop efficient CRISPR/Cas9 methods to be used in genetic engineering in filamentous fungi. CRISRP/Cas9 has been successfully used in *A. niger, A. oryzae, Aspergillus fumigatus, Aspergillus carbonarius, , Alternia alternata, Coprinopsis cinerea, Ganoderma lucidum, Ustilago maydis, T. reesei, PyrGicularia oryzae, Neurospora crassa, Penicillium chrysogenum, Phytophthora sojae, Ustilago maydis, Myceliophthora thermophilia, Beauveria bassiana and Shiraia bambusicola* with different mutation efficiencies. (Deng *et al.* 2017)

### 2.2.2 DSB repair pathways for CRISPR/Cas9 in filamentous fungi

Cells can repair double strand breaks (DSBs) naturally by non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ is further divided into classical and alternative non-homologous pathways (C-NHEJ and A-NHEJ). One subset of A-NHEJ is called microhomologous end joining (MMEJ). When Cas9 cleaves double strand of the target gene, cells have to correct the break to stay alive. Consequently, these pathways are exploited in CRISPR/Cas-based genome editing after an intentional DSB caused by Cas9. In addition, different desired results require the use of different pathways in genome editing. These pathways have different efficiencies in gene deletions, and they require different preparations. (McVey and Lee 2008; Deng *et al.* 2017)

In C-NHEJ, the DSB is repaired by joining the ends of the two strands. This leads into small insertions or deletions called indels which may result in frameshift mutations or premature stop codons disrupting the target gene. C-NHEJ pathway is predominant in *A. niger* to repair DSBs, and it has been exploited successfully in CRISPR/Cas9 facilitated gene disruptions. Downsides exist for this repair mechanism: first, indels are hard to detect if the gene disruption does not cause an obvious phenotype change. Second, the whole target sequence cannot be replaced by another sequence with C-NHEJ, opposite to HDR and MMEJ. (Al Abdallah *et al.* 2017) However, instead of replacing target sequence, DNA can be added to the DSB site through C-NHEJ. Zheng *et al* (2017) concluded that CRISPR/Cas9 deletions with C-NHEJ can be enhanced by transformation of linear DNA with selection marker in *A. oryzae*. C-NHEJ had inserted the linear DNA without any flanking sequences to DSB site with mutation rate of 68.3%. As such, any linear DNA may improve gene deletion efficacy by CRISPR/Cas9 with C-NHEJ.

While NHEJ needs no DNA as template, HDR works through homologous recombination and requires a donor DNA, such as a sister chromatin or a heterologous donor DNA that contain homologous flanking sequences around the target genomic site. When Cas9 generates a DSB, HDR pathway repairs the break using the homologous flanking sequences upstream and downstream of the target genomic site. As seen in Figure 2.7, the cleaved genomic target is replaced with the DNA between the flanking sequences. This DNA sequence can contain for example a heterologous gene that can be for example a selection marker, a gene encoding an enzyme or the same target gene containing mutations. Consequently, as HDR is very precise, it is also used to alter the target gene even with only one single nucleotide mutation. (Pohl *et al.* 2016; Al Abdallah *et al.* 2017)

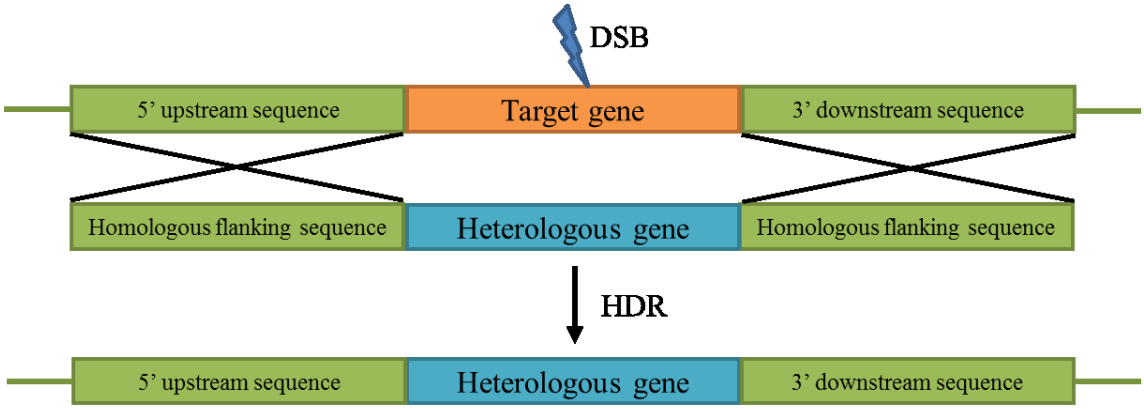



***Figure 2.7*** *Homology directed repair pathway corrects the double strand break in the genome according to an introduced donor DNA. Donor DNA attaches to the target site with homologous flanking sequences, and the enzymes in the pathway replace the target gene with a heterologous gene, for example a selection marker.*

While HDR is efficient and precise, it requires laborious preparation of a donor DNA cassettes. Homologous flanking sequences, needed for HDR in *A. niger*, are typically around 500 – 1500 bp long. As a result, the donor DNA cassette is constructed using methods such as PCR and different DNA assembly technologies. (Kuivanen *et al.* 2016; Sarkari *et al.* 2017; Nødvig *et al.* 2018) These cloning steps are time consuming and laborious and have led to studies (Nødvig *et al.* 2015; Pohl *et al.* 2016; Al Abdallah *et al.* 2017) concerning the length of flanking sequences. Applying shorter flanking sequences decreases the number of cloning steps: in donor DNA, the heterologous DNA can be amplified by PCR with extended primers that contain short flanking sequences to target gene. Consequently, flanking sequences are introduced to the donor DNA cassette effortlessly. (Al Abdallah *et al.* 2017)

In MMEJ, a DSB is repaired with donor DNA that has short, homologous flanking sequences, microhomology sequences, to the DSB site. With MMEJ, the target gene can be disrupted, modified or replaced, but this system is different to HDR. While HDR is very precise, MMEJ is prone to errors. Therefore, after MMEJ, the junctions between the target genomic site and donor DNA are mutated. On the other hand, HDR requires at least 500

bp flanking sequences in linear form while MMEJ requires only 2 – 25 bp flanking sequences. (McVey and Lee 2008) Since MMEJ is prone to errors and indels are possible, flanking sequences should not contain anything significant, nor should the sequences straight next to the flanking sequences in the donor DNA cassette.

In genome editing, C-NHEJ, HDR and MMEJ compete with each other after the Cas9 has generated a DSB. Disruption of NHEJ can improve HDR, and especially MMEJ pathway is mostly active, when NHEJ is deficient. Therefore, NHEJ pathways has been disrupted in order to enhance HDR and MMEJ pathways. (McVey and Lee 2008; Al Abdallah *et al.* 2017) For example, genes *akuB, kusA, ku70, ku80, DNA ligase IV* have been deleted, and these strains have been used for CRISPR/Cas deletion (Nødvig *et al.* 2015, 2018; Katayama *et al.* 2016; Pohl *et al.* 2016; Al Abdallah *et al.* 2017; Deng *et al.* 2017). With deficient NHEJ pathways, genes have been deleted and replaced with 100 % efficiency through homologous recombination of only 35-200 bp flanking sequences in the donor DNA cassettes (Pohl *et al.* 2016; Al Abdallah *et al.* 2017; Nødvig *et al.* 2018).

However, having deficient NHEJ pathway is not always reasonable. Previously, the deletion of genes has failed to work in Δ*kusA* strain of *A. niger* due to probable instability of the strain. (Wang 2016)On the other hand, when the most stable strains are needed for product formation, having a deficient NHEJ may not be a wise solution. During this study, Nødvig *et al.* (2018) reported that short 100 – 200 bp flanking sequences were used successfully in CRISPR/Cas9 gene replacement with working NHEJ pathway, but the donor DNA cassette was in circular form. This way NHEJ could not interfere the process by attaching to free ends of the donor DNA cassette. Unfortunately, the cloning efforts were around the same as with longer flanking sequences before when the donor DNA cassette has to be in circular form.

In 2017, Abdallah *et al*, deleted a gene from *A. fumigatus* by CRISPR/Cas9 system with a donor DNA cassette that had 35 bp or 50 bp flanking sequences to DSB site. In Δ*akuB* strain they got 97% gene replacement efficacy with either length of flanking sequences, but also 74% and 90% efficacy with 50 bp flanking sequences in strains without disrupted NHEJ pathway. In this study, missing information was the sequence of the resulting genomic junctions to indicate whether the deletion had occurred through MMEJ or HDR. In any case, donor DNA cassette with 35 and 50 bp flanking sequences replaced the target gene while NHEJ was working. (Al Abdallah *et al.* 2017) As a result, it is in high interest to study if the donor DNA cassettes containing short flanking sequences would function well in CRISPR/Cas9 gene replacements in *A. niger* with working NHEJ pathways as in *A. fumigatus*.

### 2.2.3 Delivery of Cas9 & gRNA

Cas9 and gRNA are delivered into cell's nucleus with different implementations. Multiple CRISPR/Cas9 strategies have been published, and they have their own advantages, disadvantages and deletion efficiencies. The most common strategy in filamentous fungi is to express Cas9 and gRNA from plasmids inside the cells, where coding sequences for Cas9 and gRNA are either in same or separate plasmids. Often these plasmids contain a selection marker in order to recognize and select transformants with the plasmid (and targeted deletion). Alternatively, cells are transformed with *in vitro* synthesized gRNA or purified Cas9 instead of expression. A common method is to produce Cas9 in the cells and transform the cell with gRNA. Alternative technique includes *in vitro* formed RNP complex, consisting of the Cas9 protein and crRNA:tracrRNA duplex, transformed into the target cells triggering CRISPR/Cas9 deletions. (Deng *et al.* 2017)

The transcription gRNA is most commonly under an efficient poly III promoter, such as U6. Unfortunately, efficient poly III promoters are not always available or functioning well in different filamentous fungi species. Also a poly II promoters, such as *gpdA* promoter, are possible but gRNA requires also ribozyme modification after transcription. (Deng *et al.* 2017) In order to prevent possible challenges in the expression and labor intensive cloning, an interesting alternative is to synthesize *in vitro* transcribed gRNA or order chemically synthesized crRNA and tracrRNA that are combined together into gRNA complex (Kuivanen *et al.* 2016; Al Abdallah *et al.* 2017; Zheng *et al.* 2017).

The most common way for the delivery of Cas9 in filamentous fungi is to construct and deliver a plasmid with the gene of Cas9 inside the cells. The Cas9 has been mostly expressed under constitutive *tef1* promoter. (Deng *et al.* 2017) Unfortunately, if the expression of Cas9 is continuous, the cells may be under a lot of stress and off-target mutations may occur, since there is a lot of Cas9 in the nucleus (Kim *et al.* 2015; Pohl *et al.* 2016; Korppoo 2017). Since the expression of Cas9, selection markers and gRNAs are not often beneficial after the deletion as they cause of-targets or cell stress, unstable plasmids can be used to prevent long-lasting expression. For example the replicator from A. fumigatus, AMA1, is mitotically unstable, and the plasmids containing AMA1 disappear mostly from *A. niger* transformants after CRISPR/Cas9 method has been applied. (Sarkari *et al.* 2017)

CRISPR/Cas9 system has been applied successfully in *A. niger* with different approaches (Nødvig *et al.* 2015, 2018; Kuivanen *et al.* 2016; Sarkari *et al.* 2017). In 2015, Nødvig *et al.* deleted a gene from *A. niger* with CRISPR/Cas9 using a plasmid expressing Cas9 under *tef1* promoter and terminator and gRNA under *gpdA* promoter and *tef1* terminator. Gene deletions occurred through NHEJ. Unfortunately, the method gave a much lower amount of transformants for *A. niger* than for *A. nidulans* and *A. aculeatus*. In 2017, Sarkari *et al.* succeeded in using CRISPR/Cas9 with NHEJ and HDR in *A. niger* with Cas9 and gRNA expressing plasmid in which they under the promoters *coxA* and *pmfbA*

from *A. niger* . Especially HDR succeeded with 100% deletion efficacy. (Sarkari *et al.* 2017) As for Kuivanen *et al.* (2016), Cas9 was expressed under *gpdA* promoter while the gRNA was *in vitro* transcribed and transformed into *A. niger* simultaneously with the donor DNA cassette with homologous flanking sequences. Using *in vitro* gRNA, laborious cloning steps were avoided for the construction of gRNA expressing plasmid, but the frequency of correct gene deletions in resulting colonies varied between 27.5 % and 100 depending on the target gene (Kuivanen *et al.* 2016; Wang 2016).

To prevent the time-consuming and laborious cloning process for Cas9 and gRNA plasmids, an alternative option is to assemble a Cas9 and gRNA complex *in vitro* and transform the resulting RPN-complex into the cells. CRISPR/Cas9 by transformation of RNP has been done in only a couple of fungal species mostly with polyethylene glycol (PEG) mediated transformation, but the method has been applied to mammal cells for years. (Deng *et al.* 2017) RNP-complex based gene deletion succeeded in *Penicillium chrysogenum* with HDR with 500 – 1000 bp flanking sequences (Pohl *et al.* 2016). Also a gene was replaced when *Aspergillus fumigatus* was transformed with two RNP-complexes for the single target with donor DNA containing 35 – 50 bp flanking sequences (Al Abdallah *et al.* 2017). In addition, Abdallah *et al* (2017) concluded that the amount of RNP had effect on the deletion efficacy. More there was RNP, more there were correct deletions in the transformants.

Downsides to RNP-based CRISPR/Cas9 are that there are no temporary selection available for Cas9 or gRNA delivery as for plasmid based systems, and ordered Cas9 and gRNAs are quite expensive (Pohl *et al.* 2016; Al Abdallah *et al.* 2017). One option to avoid permanent heterologous selection markers in the strains would be to use homologous selection in donor DNA cassettes, for example transformation of *pyrG,* a gene for orotidine-5'-phosphate decarboxylase, to Δ*pyrG, pyrG*imidine deficient strain. Pohl *et al.* (2016) resolved the problem of marker free selection so that they transformed AMA1 and a selection marker containing plasmid at the same time as RNP to *P. chrysogenum*. As for the second restriction, the costs per one transformation are around 165 $. An alternative option is to construct the purified Cas9 and gRNAs in one's own laboratory. (Al Abdallah *et al.* 2017) Nevertheless, the required labour force may consume the same amount or more of money than ordering the ready *in vitro* Cas9 and gRNAs from the market. As CRIPSR/Cas9 becomes more and more used and technology develops, the Cas9 and gRNAs probably, become cheaper over time.

Construction of gRNA and Cas9 expression plasmids requires a lot of time and effort, as does the forming of gRNA and Cas9 expressing strains. While Cas9 causes stress to cells and some fungi species lack effective gRNA promoters, the transformation of RNP-complexes has risen a lot of interest in CRISPR/Cas9 system due to its simplicity, portability, efficacy and decrease in off-targets. (Pohl *et al.* 2016; Al Abdallah *et al.* 2017; Grahl *et al.* 2017) Yet, the straight delivery of RNP-complex by transformation as CRISPR/Cas9 method has not been tried in industrially relevant species of *A. niger*.

### 2.2.4 Multiplex genome editing with CRISPR/Cas9 system

Often in metabolic engineering, more than one gene are deleted, modified, inserted or replaced. As a result, multiplex genome editing, also called as multiplexing, is being studied. Multiplexing is possible for example when different gRNAs are in same CRISPR array and expressed from the same plasmid (Cong *et al.* 2010). Deletion of multiple genes at the same time was first succeeded in human and murine cells, but has been tried in only two filamentous fungi. (Liu *et al.* 2015; Korppoo 2017; Nødvig *et al.* 2018) On the other hand, multiplexing has been successfully applied to many other eukaryotes, for example human cells, mice, plants and yeast. CRISPR/Cas9 system enables simple multiplexing since only design and delivery of different gRNAs are needed for multiple simultaneous gene alterations. This is not possible with old gene editing techniques like ZNFs or TALEN which include design of specific proteins for each gene resulting in difficulties in design, synthesis and delivery of genome editing agents. (Thompson *et al.* 2017)

The following transformations using CRISPR/Cas9 were done in filamentous fungi: in 2015, two genes were simultaneously deleted in *T. reesei* through CRISPR/Cas9 method with HDR resulting in 16 % frequency of double recombination (Liu *et al.* 2015). As the last study was conducted through in vivo expressed Cas9 and in vitro synthesized gRNA, RNP complex was in used also in double recombination in 2017 (Liu *et al.* 2015; Korppoo 2017) . RNP-mediated multiplexing led only to 1 – 2 % of screened colonies containing both correct replacements. The donor DNA cassette contained selection marker for screening positive clones. Multiplexing was succeeded with different crRNAs in RNP-mixes. (Korppoo 2017) Multiplex genome editing was also performed in *A. nidulans* where two genes were deleted with single stranded oligonucleotides and one gene repaired with a donor DNA cassette through HDR. Different gRNAs were expressed from plasmid. (Nødvig *et al.* 2018) Multiplexing has not been previously studied in *A. niger*. Multiple gene replacements by HDR to heterologous genes (other than selection markers) and the expression of these heterologous genes in filamentous fungi have not yet been described.

# 3. MATERIALS AND METHODS

The donor DNA (dDNA) cassette plasmids were designed and constructed for industrial relevant species of *A. niger* and *A. oryzae* since the RIB40 strain of *A. oryzae* also contained probable *gaaX*, *gaaA* and *39114* homologs according to a web tool called BLAST (National center for Biotechnology information, USA). Due to time limits, the CRISPR/Cas9 method and MA production from pectin-rich materials was studied in *A. niger*. The dDNA cassette plasmids remain to be used in possible future gene replacements in *A. oryzae*.

## 3.1 Strains, media and culture conditions

Table 3.1 contains the list of *A. niger* and *A. oryzae* strains engineered and grown in this study. The similarity of genomes between *A. oryzae* D-88349 and D-88351 with *A. oryzae* RIB40 was verified by sequencing the PCR products of *gaaX* and *pyrG* homolog and its upstream and downstream sequences. At least *gaaX* and *pyrG* were identical in both strains of *A. oryzae*. As a consequence, D-88349 and D-88351 were used to construct donor DNAs for *A. oryzae* since The RIB40 strain with available genome is patented.

***Table 3.1*** *All filamentous fungi cultivated in this study.*

| Name | Parental strain | Genetic modifications/ Applications | Reference |
|---|---|---|---|
| ***A. niger*** | | | |
| ATCC 1015 | | Wild type reference, genomic DNA | |
| Δ*pyrG* | ATCC 1015 | Deletion of *pyrG* gene | (Kuivanen *et al.* 2016) |
| Δ*gaaX* | Δ*pyrG* | Replacement of *gaaX* by *pyrG* | This study |
| *ΔgaaA-ΔgaaC$_{0\text{-}264bp}$ UDH* | Δ*pyrG* | Replacement of *gaaA* by *pyrG* and *UDH* | This study |
| *ΔgaaA-ΔgaaC$_{0\text{-}264bp}$ Δ39114 UDH* | Δ*pyrG* | Replacement of *gaaA and 39114* by *pyrG* and *UDH* | This study |
| *ΔgaaA-ΔgaaC$_{0\text{-}264bp}$ Δ39114 ΔgaaX UDH* | Δ*pyrG* | Replacement of *gaaA, 39114 and, gaaX* by *pyrG* and *UDH* | This study |
| *M895* | Δ*pyrG* | Replacement of *gaaA* by *UDH* | (Mojzita *et al.* 2010) |
| *M1767* | | Replacement of *gaaA* and *39114* by *pyrG* and *UDH* | (Kuivanen *et al.* 2016) |
| ***A. oryzae*** | | | |
| D-88349 | | Extraction of genomic DNA | VTT collection |
| D-88351 | | Extraction of genomic DNA | VTT collection |
| D-88353 | | Extraction of genomic DNA | VTT collection |

All used media are listed in the table 3.2. *E. coli* TOP10 cells (B-3197 VTT culture collection) were grown on Lysogeny Broth (LB) medium with 25 µg/ml chloramphenicol during the construction of entry vectors and with 100 µg/ml ampicillin during the construction of donor DNA cassettes for selection. The cultivations were kept in 37ºC overnight first as plates and second as 4 ml cultivations in 700 RPM in 24-well plate or as 5 ml cultivations in 220 RPM in glass tubes.

***Table 3.2*** *All medias and plates applied in this study*

| **Media** | **Abbreviation** | **Usage** |
|---|---|---|
| Lysogeny Broth + 25 µg/ml Chloramphenicol | LB+CAM | *E. coli* cultivation |
| Lysogeny Broth + 10 µg/ml Ampicillin | LB+AMP | *E. coli* cultivation |
| Yeast Peptone medium, 3% gelatin, pH 7 | YP+G | *A. niger* precultivation |
| Mineral medium + 1 % xylose | MM+XY | *A. niger* GaaA activity analysis |
| Synthetic complete medium without uracil | SCD-URA | *A. niger* GaaA activity analysis |
| Yeast peptone medium + 20 g/l galacturonic acid | YP+GA | *A. niger,* MA production |
| Yeast peptone medium + 20 g/l galacturonic acid + 10 g/l | YP+GA+Glu | *A. niger,* MA production |
| Yeast peptone medium + 20 g/l citrus pectin | YP+Pectin | *A. niger,* MA production |
| **Plates** | **Abbreviation** | **Usage** |
| Lysogeny Broth + 25 µg/ml Chloramphenicol | LB+CAM | Entry vector construction |
| Lysogeny Broth + 10 µg/ml Ampicillin | LB+AMP | Deletion cassette construction |
| *A. niger* transformation plates | SCD-URA | *A. niger* transformation |
| Potato extract | PD | *A. niger* purification through one single colony |

*A. niger* were grown in 28 ºC for 3-4 days. *A. niger* was grown on SCD-URA transformation plates after transformations and for strain purification, by turns, with potato dextrose (PD) plates. In submerged cultivations, *A. niger* grew in various YP cultivations in 220 or 800 RPM in 28 ºC. LB media contained 5 g/l yeast extract and 10 g/l tryptone while all YP media contained 10 g/l yeast extract and 20 g/l peptone. In the cultivation of A. niger for GaaA activity analysis, mineral media included $NaNO_3$, KCL and $KH_2PO_4$ and 20 g/l agar. PD plates contained 39 g/l potato dextrose agar. In *A. niger* transformations, SCD-URA plates contained 6.7 g/l yeast nitrogen base supplemented with 20 g/l D-glucose, 1.2 M D-sorbitol and 20 g/l agar.

## 3.2 Preparation of DNA constructs

Donor DNA cassettes were constructed within plasmids with Modular Cloning (MoClo) system (Lee et al, 2015) using Golden Gate assembly (Engler *et al.* 2008). Golden Gate assembly method allows one-pot cloning through unique cohesive ends resulting from the cut of type II restriction enzymes. In addition, this cut site is outside the recognition site which is often a desired feature providing flexibility (Engler *et al.* 2008; Lee *et al.* 2015). MoClo was applied because it is a standardized method to make cassette plasmids and multigene plasmids with predesigned entry vectors. Moreover, while DNA parts are cloned as MoClo parts, they are suitable for later use as well.

First, all new parts needed for the dDNA cassette plasmids were amplified with PCR with KAPA Hifi PCR Kit (KAPA biosystems., USA) and cloned into entry vectors (pytk001). All primers are listed in Appendix A. Second, parts were digested from entry vectors with BsaI and combined to form dDNA cassette plasmids. The linear dDNA cassettes were released from the plasmids with NotI and finally the deletions were made with CRISPR/Cas9 method based on homologous recombination. Appendix B lists all plasmids used in this study.

For the transformation of *Escherichia coli* cells, 1 µl of the product of the Golden Gate reaction was pipetted into 40 µl of *E. coli* TOP10 electrocompetent cells on ice. The mixture was transferred to the bottom of an ice-cold cuvette and cells were given a electric shock of 2,5 volts with Bio-Rad Gene Pulser and Pulse Controller. The SOC solution (1 ml) was added to the cuvette and the mixture was transferred into 5 ml tube. The tube was incubated in room temperature for one hour. Different amounts of transformation mixture, 10 – 100 µl, were plated on LB plates containing chloramphenicol or ampicillin (see 3.1 ). Plates were incubated over night at 37ºC.

### 3.2.1 Construction of entry vectors

All flanking sequences and primers were designed with Geneious ver. 10.1.3 (Biomatters Ltd. , New Zealand) to be suitable for the MoClo system. Homologous flanking sequences for *gaaX* (NRRL_08194) were designed to be 100 bp, 500 bp, 1000 bp and 1500 bp long for *A. niger* and 1500 bp long for *A. oryzae*. For *gaaX* in *A. niger,* upstream flanking sequences had the same reverse primer and downstream flanking sequences had same forward primer to ensure the reliability when comparing the consequences of flanking sequence's length. Flanking sequences for *gaaA* (NRRL_5650) and *39114* (NRLL_*39114*) deletions both in *A. niger* and *A.oryzae* were designed to be between 1200 bp and 1500 bp. For *gaaA* in *A. niger* this posed a challenge since its upstream contained numerous BsaI sites, and finally the upstream flanking sequence had to be designed so that it also deleted the first 264 bp from the next gene's, *gaaC*'s, open reading frame. As a result, *gaaC* was targeted to be disrupted simultaneously with *gaaA*.

The flanking sequences, the *pyrG* in *A. oryzae* and the promoter of *gaaB* were amplified from the genomic DNA (wild type *A. niger* and D-88349 *Aspergillus flavus var. oryzae)* and *UDH* from *UDH*-plasmid in PCR using KAPA Hifi PCR Kit and Mastercycler or Mastercycler Pro S (Eppendorf, Germany). PCR products were purified with Qiaquick PCR purification kit (Qiagen, Germany) and inserted to MoClo entry vector (pytk001) using BsmBI, T4 DNA ligase and Golden gate protocol (Engler *et al.* 2008) in Mastercycler or Mastercycler Pro S. (Eppendorf). Figure 3.1 represents the entry vector with 1500 bp upstream sequence from *gaaX*. Entry vectors were transformed into *Escherichia coli* using electroporation method. The entry vectors were extracted from 4 or 5 ml cultivations using Miniprep kit (Thermo Scientific, USA). Successful cloning was confirmed with a digestion by BsaI or with a colony PCR with DreamTaq Green DNA Polymerase protocol (Thermo Scientific). The size of the PCR product was verified in a gel electrophoresis comparing with GeneRuler 1 kb ladder (Thermo Scientific) and Gel Doc XR+ Imager (Bio-Rad Laboratories, USA). DNA staining was done with ethidium bromide. The entry vectors were verified to be flawless using the Sanger sequencing services from GATC Biotech. The correct sequences of the entry vectors were confirmed using the Geneious software.

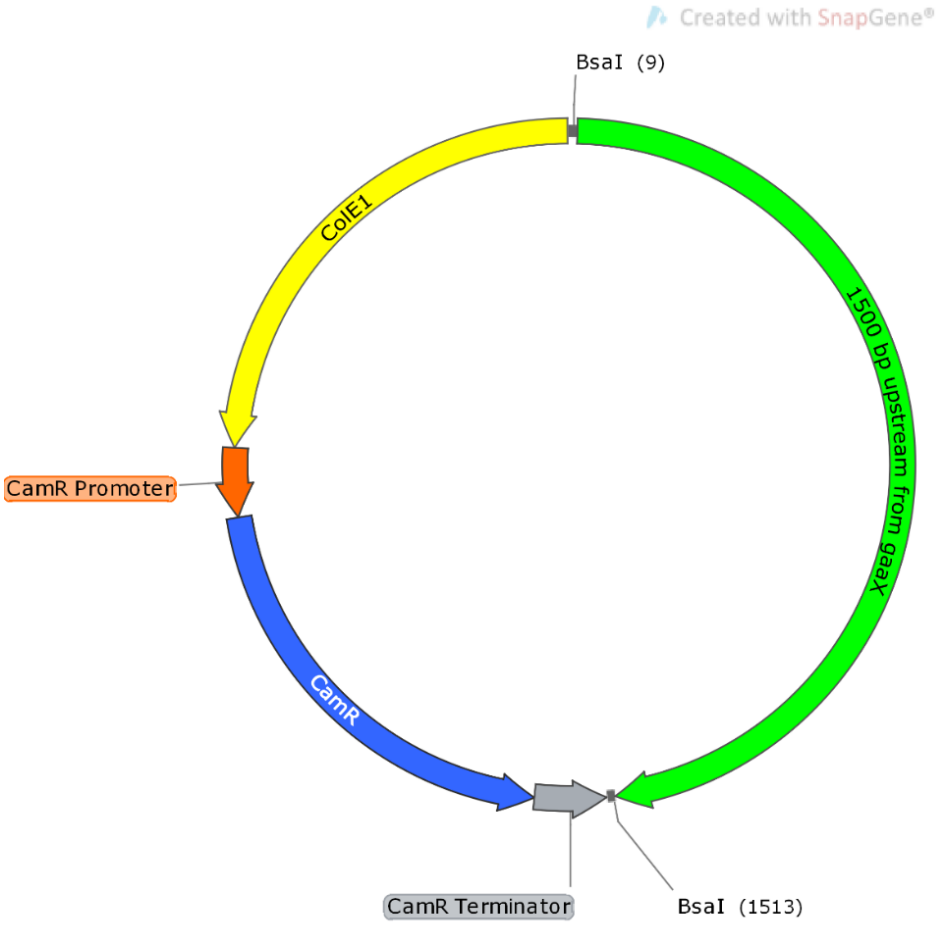



***Figure 3.1*** *Pytk001 entry vector (3166 bp) with 1500 bp sequence upstream from gaaX.*

### 3.2.2 Construction of donor DNA cassettes

The dDNA cassette plasmids were constructed with BsaI, T4 DNA ligase and suitable entry vectors. After this, the dDNA cassette plasmids constructed according the MoClo protocol were transformed into *E. coli* by the electroporation method. The plasmids were extracted with GeneJet Plasmid Miniprep kit (Thermo Scientific). The construction of the dDNA cassettes was confirmed to be correct by NotI digestion leading to two parts: the donor DNA cassette and the *E. coli* origin of replication with ampicillin resistance gene as seen in Figure 3.2.

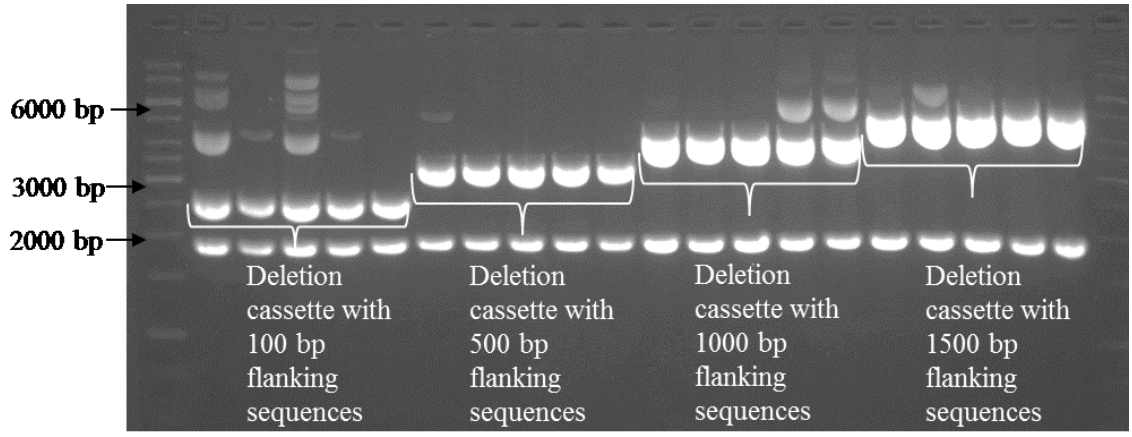



***Figure 3.2*** *Deletion cassette vectors were digested by NotI resulting in linear donor DNA cassettes and separate linear DNAs with ampicillin resistance marker gene and E. coli origin of replication.*

Each dDNA cassette vector had two flanking sequences, one upstream and one downstream flanking sequence around the target gene, to enable the deletion and replacement of the target gene by homologous recombination. Other parts used were taken from ready entry vectors listed in Appendix B. The dDNA cassettes for the first single deletion of *gaax* included only *pyrG* as selectable marker whereas dDNA cassettes for multiplexing to construct *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ *Δ39114 ΔgaaX* also consisted of *UDH* expression under *gaaB* promoter. All deletion cassettes and the entry vector used in them are listed in Appendix B.

The dDNA cassettes were digested with NotI from the constructed dDNA cassette plasmids and concentrated by ethanol precipitation. After the 2-hours-lasting digestion at 37ºC, 800 µl of absolute ethanol and 32 µl of 3 M Sodium Acetate were added. The mixture was incubated at -20ºC for 20 min and centrifuged at 4ºC for 10 min at full speed. The supernatant was pipetted away and the pellet was washed with 200 µl of 70% ethanol. The mixture was centrifuged in room temperature for 5 min. The supernatant was repeatedly pipetted away and the pellet was dried from the ethanol before adding 20 µl of STC buffer. The concentration of the DNA was adjusted to 1000 ng/µl when one gene was to be deleted and 2000 ng/µl when two or three genes were to be deleted.

## 3.3 Targeted gene replacements in *A. niger*

Two specific crRNAs were designed for each gene deletion with Geneious software (Geneious) and verified with a web tool called CHOPCHOP V2 (Montague *et al.* 2014; Labun *et al.* 2016). Specific crRNAs were designed to target the middle of the gene. Cas9 protein, crRNA and tracrRNA were ordered from Integrated DNA Technologies (IDT, USA). The generation of Cas9:crRNA:tracrRNA ribonucleoprotein (RNP) complexes was based on the same company's protocol to assemble RNP-complexes: 1 µl of specific crRNA (100 µM) and 1 µl of general tracrRNA (100µM) were joined and diluted to 1 µM with Nuclease free duplex buffer (IDT). The mixture was incubated at 90°C for 5 min to form crRNA:tracrRNA duplexes, cooled down to room temperature and stored at -20ºC. The RNPs were formed when Cas9 (61µM) and crRNA:tracrRNA duplex solution (1 µM) were joined and diluted with Cas9 enzyme working buffer (20 mM HEPES, 150 mM KCl, pH 7.5). The concentration of RNP was set to 60 nM, 540 nM or 1080 nM with Cas9 depending on the phase of optimization.

First, the transformation method in 3.3.1 was tested whether the transformation of *A. niger* could be done in the first place in 96-microwell format or not. This was done by transformation of *pyrG* into Δ*pyrG A. niger*. The functionality of *pyrG* from *A. oryzae* was simultaneously checked with same protocol to ensure its capability of working as a selection marker in *A. niger*. Genes *gaaX*, *gaaA* and *39114* were replaced by more downscaled CRISPR/Cas9 method described in 3.3.2. The method was based on homologous recombination seen in Figure 3.3.

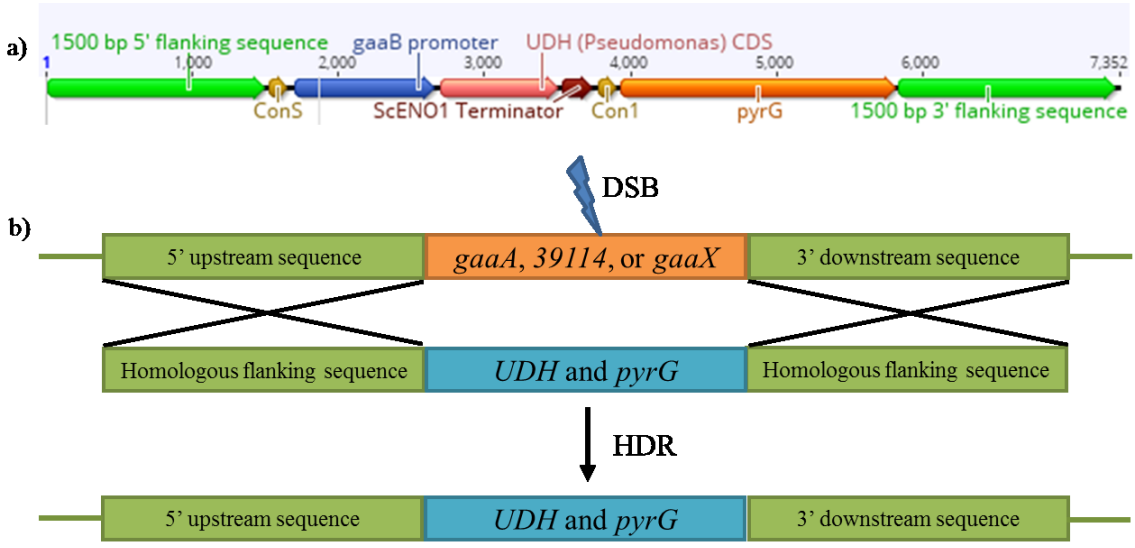



***Figure 3.3*** *a) The structure of the donor DNA cassette for gaaA, 39114 and gaaX to be replaced by UDH and pyrG b) Genes gaaA, 39114 and gaaX are replaced by UDH and pyrG by homologous recombination after DSB caused by Cas9.*

The *gaax* was replaced with *pyrG* in Δ*pyrG* strain of *A. niger* while the CRISPR/Cas9 method was tested with co-transformation of RNP and dDNA cassette including *pyrG*.

After the verification of method's functionality, the CRISPR/Cas9 method was downscaled more and optimized with respect to the length of the homologous flanking sequences and to the amount of RNP. The developed method was repeated and its efficiency was verified in the replacement of *gaaA* with in Δ*pyrG*. The new protocol was tested for deleting multiple genes simultaneously. Multiplexing was performed as deletion of two genes, *gaaA* (*gaaA:gaaC*$_{0\text{-}264bp}$) and *39114,* and as deletion of three genes: *gaaA (gaaA:gaaC*$_{0\text{-}264bp}$,) *39114* and *gaaX*.

### 3.3.1 Transformation of *A. niger* by protoplast method

Transformation of *A. niger* was based on the protoplast method described earlier by Yelton *et al.* 1984). 500 µl of spores were inoculated into 125 ml of YP with 3 % of gelatin and 5% xylose in 250 ml sterile Erlenmeyer flask and grown at 28ºC in 220 RPM overnight. Next day the cultivation was filtered into a 500 ml Erlenmeyer flask with vacuum. The germinated spores were washed first with 37ºC warm sterile water and then with cold KMC buffer (1 M KCl, 25 mM $CaCl_2$, 10 mM Tris-HCl, pH 5.8). The filtered mycelia were resuspended into 50 ml of cold KMC buffer with 10 mg/ml caylase and incubated at 30ºC in 80 RPM for 3 hours.

After individual protoplasts could be seen under a microscope, they were filtered into a cold 250 ml Erlenmeyer flask first without vacuum and then with vacuum through 2 layers of thin Miracloth (Calbiochem, Germany) prewet before with cold KMC buffer. Protoplasts were kept on ice for the rest of the procedure. The filtered protoplast solution was transferred into 50 ml sterile falcon tube and centrifuged in 1500g at 4 ºC for 4 min. The supernatant was poured off, the pellet was resuspended into 20 ml of cold KMC buffer, and the solution was centrifuged again as before. The supernatant was poured off, the pellet was resuspended into STC solution (1.33 M sorbitol, 10 mM Tris-HCl, 50 mM $CaCl_2$, pH 8.0) and the same centrifugation was repeated. The supernatant was poured off and the pellet was resuspended into remaining STC buffer (around 200 µl). Finally, the number of protoplasts was measured with Cellometer Auto M10 (Nexcelom Bioscience LLC). Finally, protoplasts was diluted to either $1*10^8$ per ml with STC buffer.

1 µl DNA (1000 ng) was filled up to 10 µl of STC buffer and joined with 10 µl of protoplasts and 10 µl of transformation solution (25% PEG 6000, 50 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5) in a 96-well-plate. This mixture was incubated 20 min on ice before adding 200 µl of same transformation solution. The mixture was kept in room temperature for 5 min and 800 µl of STC solution added. The total 1000 µl solution was mixed gently by pipetting, transferred on SCD-URA *A. niger* transformation plates and spread quickly by moving the plate. The plates were transferred to grow at 28ºC for 2-6 days.

### 3.3.2 CRISPR/Cas9 replacements by transformation of RNP

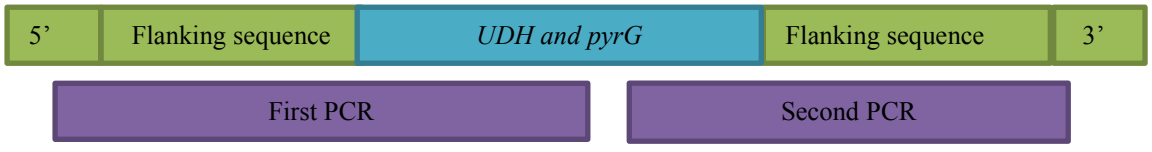
The deletion of genes by transformation of RNP was performed as described above apart from a couple of exceptions. The DNA -mixture consisted of 1000 ng of donor DNA cassette and 0.5 – 9 µl of Cas9-crRNA-tracrRNA complex filled up to 10 µl with STC buffer. At first, 10 µl of $1*10^8$ protoplasts/ml were used for deletions. As a result, single colonies were hard to pick and many colonies grew on control plates. During the transformation, top agar was difficult to spread on the plates since it solidified quickly. As a consequence to these challenges, 10 µl of $1*10^7$ protoplasts/ml, 57 µl of transformation solution, 113 µl STC solution and none of top agar was used in the next RNP transformation. The total 200 µl solution was mixed gently by pipetting, transferred on transformation plates and spread with 10 – 20 glass beads.

Downscaled method was used to study the effect of the amount of RNP-complex and the length of homologous flanking sequences while replacing *gaaX* with *pyrG*. Multiplexing was performed as described above, but 10 µl of DNA-RNP-mixture included equally 2.5 µl of RNP-complexes (1080 nM) and 1000 ng of tailored donor DNA cassette for each gene. While multiplexing was performed, it was studied, how the use of different crRNAs or multiple crRNAs for the same gene effect the deletion efficacy. In the case of two crRNA for the same gene, 1.25 µl of different RNP-complexes (1080 nM) were combined.

### 3.3.3 Verification of correct replacements

After 3 – 4 days of cultivation, the number of *A. niger* colonies was calculated from the transformation plates. Single colonies were removed with a pipette tip and put to grow on a PD plate. The cells of one colony were lysed when same pipette tip was pushed to the bottom of the well in a 96-microwell PCR plate with 20 µl of Phire Plant PCR buffer. Lysates were kept in 100 rpm for 2 hours. Colony PCR was done with Phire Plant Direct PCR Kit (Thermo Scientific) to check whether the gene had been replaced correctly with donor DNA by homologous recombination or not. Replacements were verified with 3 different PCR's: two out of the three PCRs amplified products with a primer outside the flanking sequence of the genomic target site and with a primer in the heterologous gene of the dDNA cassette as shown in Figure 3.4. The third PCR amplified the replaced (deleted) gene so it should not have given any product. PCR products were checked with agarose gel electrophoresis as seen in Figure 3.5.

a)




b)

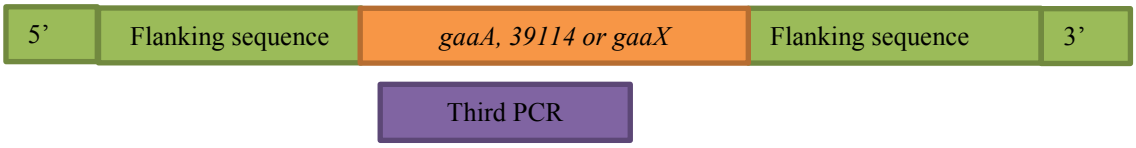



***Figure 3.4*** *a) PCR reactions to confirm the correct deletion and replacement of gaaA, 39114 or gaaX. The length of the PCR products varied between different genes but the first PCR product was about 1500 bp and the second PCR product was about 2000 bp b) Extra PCR reaction confirmed that the gene was not present anymore in the genome. If the gene had not been deleted, the PCR product was about 700 bp long.*

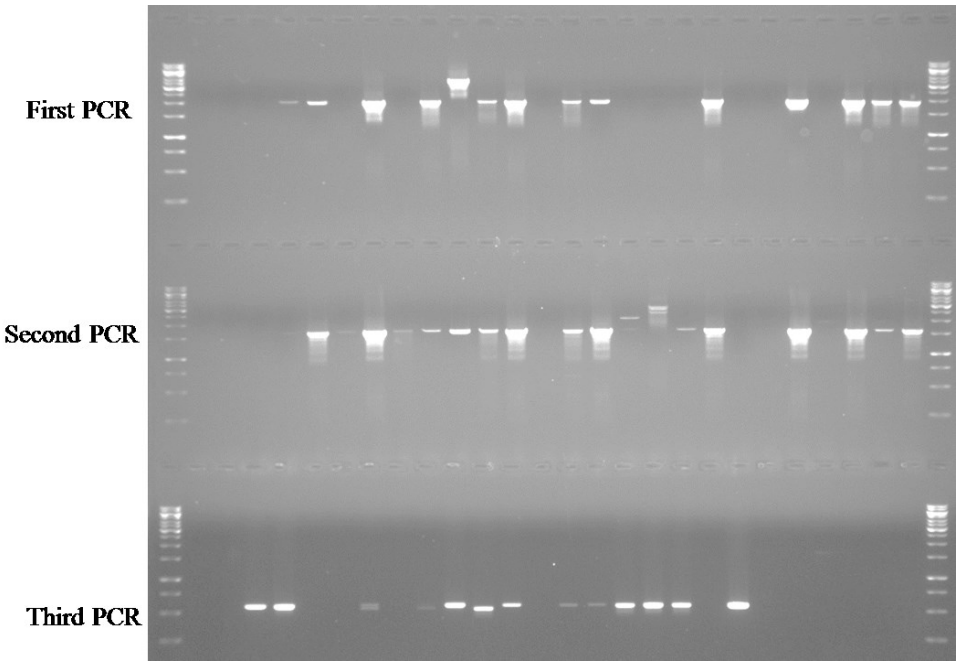



***Figure 3.5*** *Three PCR reactions to confirm the correct deletion and replacement of genes. If the first and second PCR amplified correct product and third gave no product, the gene was replaced correctly with UDH and pyrG.*

Correct colonies were picked from the SCD-URA plates and grown on their own PD-plates in 28°C. After 3-4 days cultivation 4 ml of spore stock solution (20% glycerol, 0.08% Tween and 0.025 M NaCl) was pipeted to each plate and spores were resuspended into the solution and filtered through cotton wool. The spore suspension was diluted 1:1000000 with sterile water and 10 µl of the dilution was spread on SCD-URA plates. Single colony was picked from each plate and put to grow on a PD-plate. After 3-4 days cultivation, spores were resuspended and filtered again. Finally, single spores with the exact same genome were stored in -80ºC for later cultivations.

Since colony PCR is not the most trustworthy method for screening, the genomic DNA of possible 3 *ΔgaaA-ΔgaaC$_{0\text{-}264bp}$ Δ39114 UDH* and 3 *ΔgaaA-ΔgaaC$_{0\text{-}264bp}$ Δ39114 ΔgaaX UDH* strains was collected by phenol-chloroform method described in (Graaff *et al.* 1988). Before the DNA extraction, lysis of mycelia was performed with 500 µl glass beads (0.5 mm), 300 µl of TE-buffer (0.01 M Tris, 0.001 EDTA, pH 8) and Precellys 24 Homogenizer (Bertin Instruments) in 5500 rpm for 30 s two times. Finally. Colonies with correct replacement were verified through KAPA2G robust PCR (KAPA biosystems) using genomic DNA. Once colonies with correct replacements were verified, upstream and downstream sequences of each deletion site were amplified with KAPA Hifi PCR kit. Correct PCR products were extracted from the gel with Qiagen gel extraction kit and sent to sequencing (GATC biotech) to confirm flawless gene replacement with dDNA cassette by HDR.

## 3.4 Mucic acid production

After the development of a new CRISPR/Cas9 method for *A. niger*, *gaaX* replacement (deletion by HDR) was verified to influence on the activity of enzymes related to pectin catabolism. Since galacturonic acid reductase was more active in Δ*gaaX A. niger* than in wild type, the study was continued with the combined deletion of *gaaX, 39114* and *gaaA* (presented 3.3). After, submerged cultivations of 4 ml were carried out in different media. New and old strains were compared with each other respect to MA production and GA consumption by HPLC.

### 3.4.1 Galacturonic acid reductase assay

The effect of *gaaX* deletion was studied with galacturonic acid reductase, GaaA, activity assay based on the method described by Kuorelahti *et al.* (2005). 500 µl of *A. niger* spores were inoculated to 125 ml of YPD with 3 % gelatine and grown over night at 28ºC in 220 RPM 250 ml Erlenmeyer. The cultivation was filtered through one glass microfibre filter with vacuum. The layer of mycelia was washed first with 37ºC warm sterile water and divided into three pieces. One piece was resuspended in 50 ml MM + xylose and other in 50 ml SCD-URA. Solutions were incubated at 28ºC in 220 rpm for 4 hours and filtered again as before. About half of the mycelia layer was transferred into 2 ml tube with 1 ml of $Na_3PO_4$ (0.1 M, pH 7), 500 µl of glass beads (0.5 mm) and 80 µl of 1×EDTA free Complete Protease Inhibitor (Roche, Switzerland). Final volume was around 2 ml. Mycelia were lysed with Precellys 24 Homogenizer (Bertin Instruments, France) in 5500 rpm for 30 s three times. Between the steps of homogenization, tubes were kept on ice for 5 min. After the lysis, the solution was centrifuged at 4 ºC for 10 min in 14000 rpm and the supernatant was removed into another tube. The amount of protein in the supernatant was tested with Bradford protein assay (Bio-Rad Laboratories ).

Spectrophotometer was blanked with a solution that contained 10 µl of cell extract, 10 µl of NADPH and 880 µl of $Na_3PO_4$. After the absorbance was settled at 340 nm, 100 µl D-galacturonic acid (250 mM) was added to the cuvette and the decrease of the absorbance was observed for 3 min. The decrease in NADPH absorbance indicates the activity of GaaA when D-galacturonic acid is reduced into L-galactonic acid. The *gaaA* is transcribed 76-fold in Δ*gaaX* compared to the wild type strain according to Niu *et al.* (2017). Here the enzyme's activity was tested and proved to be higher in Δ*gaaX* than in wild type of *A. niger*

### 3.4.2 Cultivations for mucic acid production

New strains *ΔgaaA-Δ*$gaaC_{0\text{-}264bp}$ *Δ39114 ΔgaaX UDH, ΔgaaA-Δ*$gaaC_{0\text{-}264bp}$ *Δ39114 UDH* and *ΔgaaA-Δ*$gaaC_{0\text{-}264bp}$ *UDH* as well as the old strains M1767, M895 and *A. niger* ATCC1015 were cultivated in 4 ml submerged cultivations in different media based on a method described in (Wang 2016; Paasikallio *et al.* 2017). As precultivations, 500 µl of spore suspension were inoculated into 125 ml YP with 3% gelatine and incubated at 28 ºC in 220 rpm. After a 21-hours precultivation, the mycelia were filtered through a sterile glass microfibre filter with vacuum and washed with sterile water. The mycelia were divided into three parts, weighed and resuspended into YP-GA, YP+GA+Glu and YP+Pectin to final concentrations of 6 g wet cell mass/l. Each cell suspension (500 µl, 0.03 g wet cell mass) was inoculated into 3,5 ml of YP-GA, YP-GA+Glu or YP+Pectin in 24-well plates. All 6 strains were cultivated as three replicates in three different media with initial biomass of 7.5 wet cell mass g/l. The cultures were incubated at 28 ºC in 800 rpm for 1, 2 and 5 days since every time point had its own cultivations to avoid the change of the culture volume.

### 3.4.3 HPLC analysis

Samples were diluted as 1:5 after 24 hours and 48 hours and as 1:10 after 120 hours with 5 mM $H_2SO_4$ and incubated at 100ºC for 1 hour. Consequently, the MA was supposed to dissolve entirely from the surface and inside of the cells. Sample handling was based on the protocol used by Paasikallio *et al* (2017). Samples were cooled down to room temperature, and 200 µl of each sample was filtered through 96 wells Multiscreen HTS DV Filter Plate (0.65 µm Hydrophilic Low Protein Binding, Durapore® Membrane, Merck, Germany).

The production of MA and the amount of GA was analyzed with high performance liquid chromatography (HPLC) using Animex Fast Acid column (100 mm by 7.8 mm, Biorad Laboratories) linked to Animex HPX-87H organic acid analysis column (300 mm by 7.8, Biorad Laboratories). $H_2SO_4$ (5 mM) was used as an eluent with flow rate of 0.5 ml/min while columns were maintained at 55 ºC. Detection was performed by a Waters 2489 UV/visible dual wavelength UV (210 nm) detector (Waters, USA).

# 4. RESULTS

In this study, the deletion of *gaaX*, *gaaA* and *39114* and the introduction of *UDH* to these genomic target sites were carried out by using the developed CRISPR/Cas9 method. Herein, the correct deletion means that the gene was replaced correctly by the donor DNA. In the method, transformation of *in vitro* assembled ribonucleoprotein complex (RNP) of crRNA/tracrRNA and Cas9 was used instead of *in vivo* expression from a plasmid. Double strand breaks at the target loci generated by RNPs were repaired through homologous recombination by using a donor DNA cassette containing *UDH* expression cassette. The developed CRISPR/Cas9 method was also downscaled enabling high-throughput workflows, and the RNP and the protoplast concentrations were optimized for the protocol. In addition, the effect of the lengths of the homologous flanking sequences to the deletion efficacy was studied. Finally, the resulting *A. niger* strain Δ*gaaA*-Δ*gaaC*$_{0\text{-}264}$-Δ*39114* Δ*gaaX UDH* was tested and compared to previously engineered strains respect to the production of MA.

## 4.1 CRISPR/Cas9 in *A. niger* based on transformation of RNP

This is the first description of a deletion using CRISPR/Cas9 method by transformation of RNP-complex from *A. niger*. Method was tested, downscaled and optimized regarding the amount of protoplasts and RNP-complex, the length of homologous flanking sequences and the use of different gRNAs. Correct gene replacements were analysed with a colony PCR. The correct replacement refers to a desired gene deleted by correct introduction of donor DNA cassette into the target locus through HDR. Correct replacement of genes with homologous recombination had occurred when PCR reactions targeting fragments at the 3' and 5' ends of the target genomic site gave correct bands while PCR targeting the gene itself did not result in a product.

Incorrect results appeared in different combinations in the PCR results. Some samples showed that homologous recombination had succeeded in first and second PCR reactions but also *gaaX* was amplified in 3$^{rd}$ reaction. In addition, some colonies gave correct band only for 3' PCR or 5' PCR and even some unexpected bands appeared in some of the colony PCRs. Last and quite peculiar issue was that some colonies gave no amplification at all from either of the 3 reactions. These results might have been due to insertion of an inverted the donor DNA cassette or other unexpected rearrangements at the DSB site. Many of the colonies that gave blank results, amplified PCR products later when the colony PCR was repeated. As a consequence, blank results could not be relied on since their targeted genes could have been either way correctly deleted or not. Unfortunately, this and the lack of dense colonies on the transformation plates, resulted in a decrease of the number of screened colonies.

### 4.1.1 Downscaling *of A. niger* transformation and CRISPR/Cas9

Replacement (deletion) of *gaaX* functioned with CRISPR/Cas9 by transformation of specific donor DNA cassette and RNP designed for *gaaX*, but the transformation of 1 million protoplasts (10µl of 1*10^8 protoplasts/ml) gave too much colonies as seen in Figure 4.1. Since the control plates had also a lot of colonies, the amount of protoplasts was decreased ten-fold. The method was even more downscaled and worked without using of top agar while optimizing other aspects. Finally, the replacement of genes by CRISPR/Cas9 with RNP transformation could be done in regular 96-microwell-plate in 200 µl volume and one 125 ml cultivation of *A. niger* could give protoplasts for even hundreds of transformations.

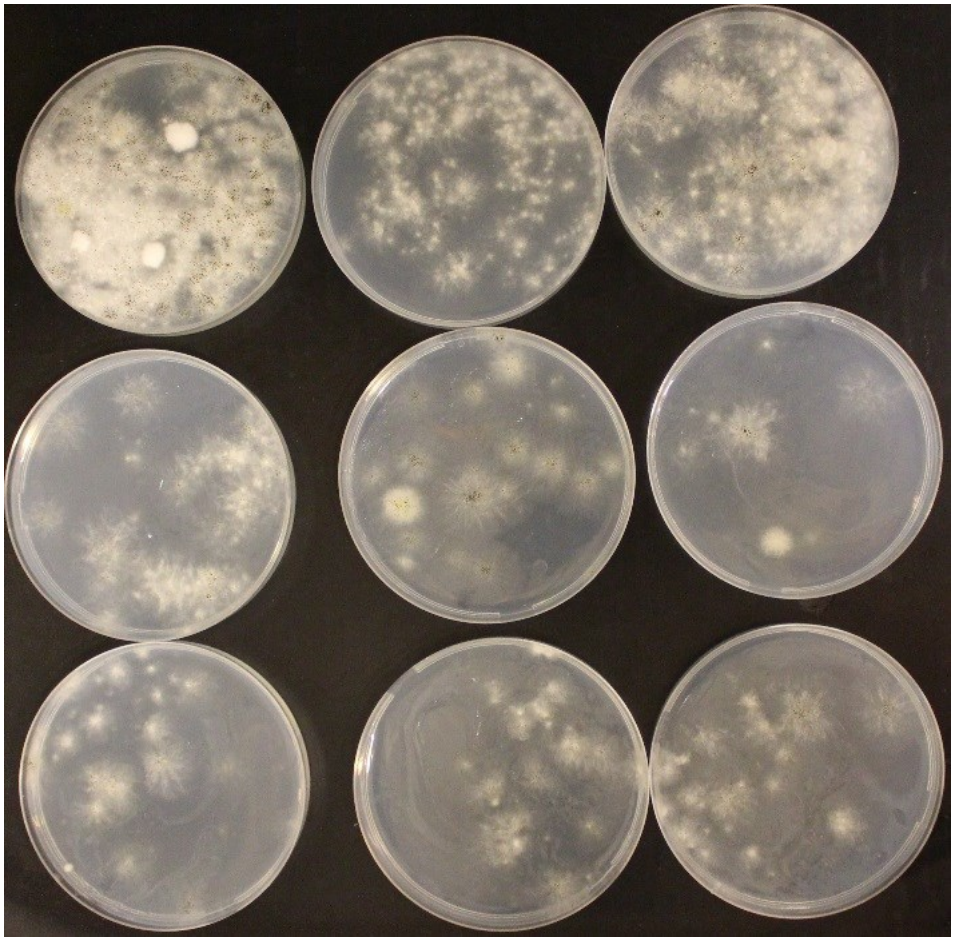


***Figure 4.1****The use of 1*10$^6$ protoplasts per transformation resulted in a lot of background on control plates situated at the last row*

### 4.1.2 Amount of RNP-complex

Figure 4.1 presents the *A. niger* transformation plates using CRISPR/Cas9 technology by transformation of RNP and of a donor DNA cassette for *gaaX* with 1500 bp long homologous flanking sequences. Table 4.1 gathers the number of colonies in transformation plates, the number of single colonies that was screened and correct deletions. Different

amounts of RNP-mix resulted in a different number of colonies and correct deletions amongst the colonies.

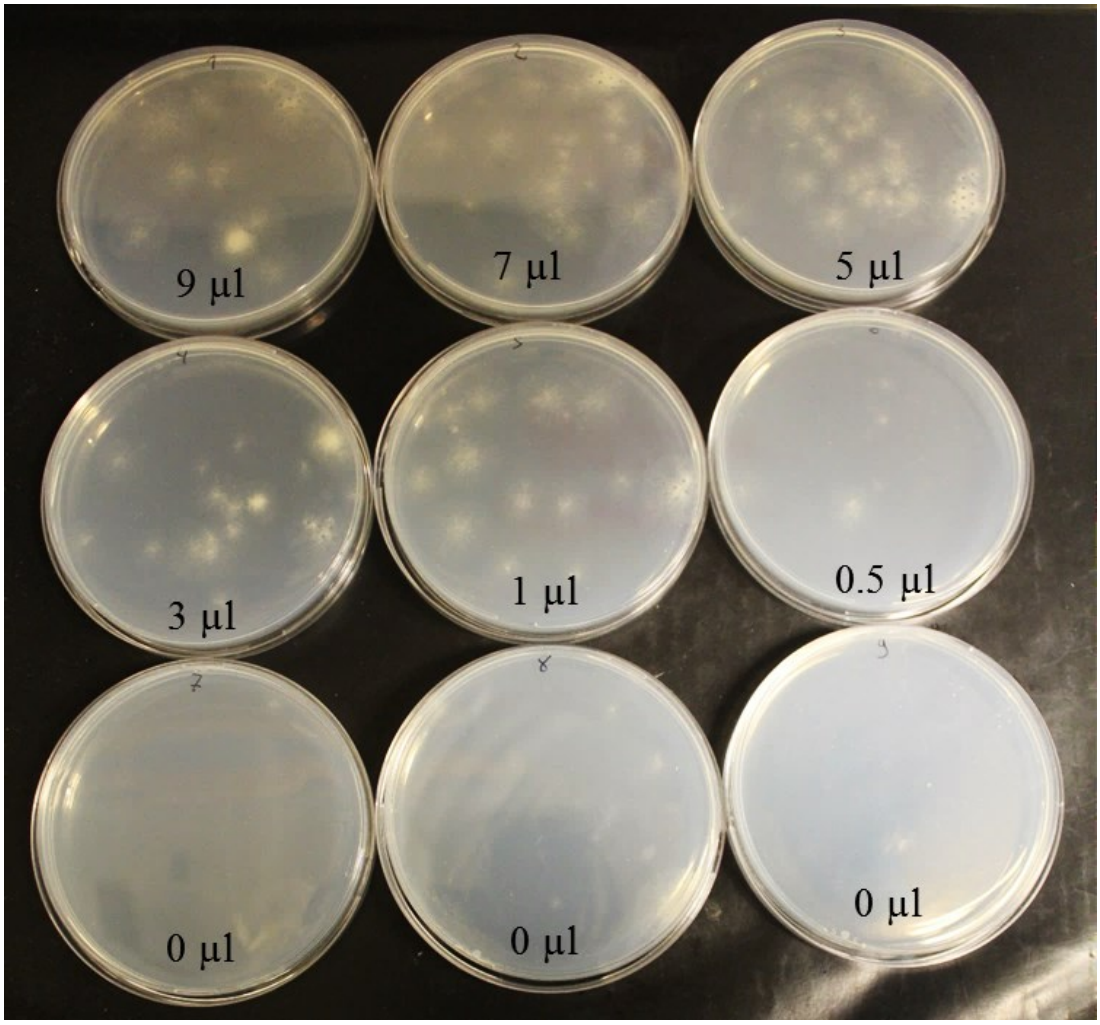



***Figure 4.2*** *In CRISPR/Cas9 method, the amount of RNP (µl) affected the number of colonies on transformation plates.*

***Table 4.1*** *The effect of the amount of RNP (µl) to the number of colonies on transformation plates and to the number of colonies with a correct deletion and replacement of gaaX.*

| **Homologous sequences (bp)** | **RNP (µl)** | **Colonies** | **Screened** | **Correct deletion of *gaaX*** |
|---:|---:|---:|---:|---:|
| 1500 | 9 | 13 | 7 | 3/7 |
| 1500 | 7 | 24 | 6 | 4/6 |
| 1500 | 5 | 26 | 5 | 5/5 |
| 1500 | 3 | 21 | 5 | 4/5 |
| 1500 | 1 | 15 | 12 | 9/12 |
| 1500 | 0,5 | 4 | 2 | 0/2 |
| 1500 (4 plates) | 0 | 12 | 4 | 0/4 |

By using 9 µl of RNP-mix, there were only 13 colonies which had less frequently correct deletions of *gaaX* than by using 7 µl or 5 µl. The use of 5 µl of RNP-mix resulted in the highest number of colonies and in the highest replacement efficiency. When less than 5 µl of RNP was used, the number of colonies and the deletion efficiency dropped gradually until 0.5 µl of RNP was not a sufficient amount to delete and replace *gaaX* correctly. As anticipated, without RNP, the transformation of donor DNA cassette gave very few colonies and in addition, the deletion and replacement of *gaaX* had not worked through HDR without the specific DSB done by Cas9 to induce HDR pathway.

### 4.1.3 Homologous flanking sequences and gRNAs

The effect of flanking sequences' length was studied with the replacement of *gaaX*. The flanking sequences affected on the number of colonies and correct *gaaX* replacements as seen in Figure 4.2 and Table 4. Homologous recombination with 1500 bp and 1000 bp homologous flanking sequences gave more colonies and more correct deletions amongst the colonies than the use of 500 bp or 100 bp flanking sequences in the donor DNA cassette. The transformation with donor DNA cassettes with 1500 bp or 1000 bp flanking sequences combined with 5 µl of RNP-complex resulted in 100 % correct replacements in transformants.

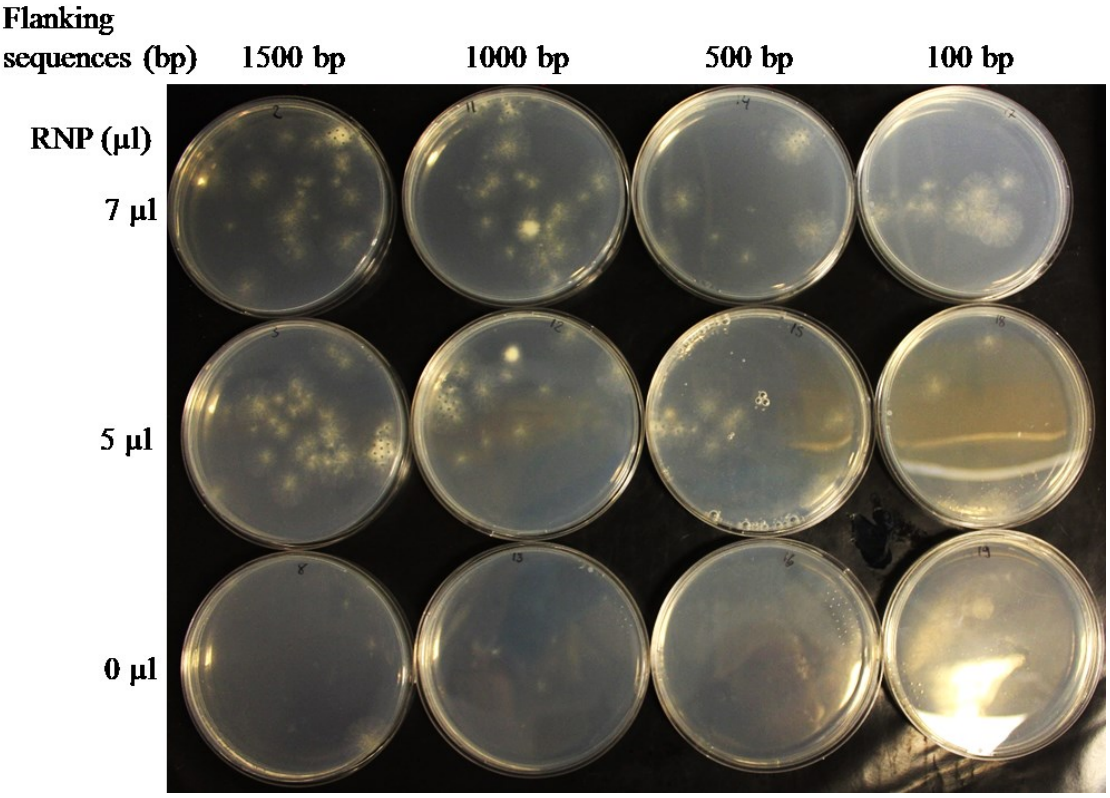



***Figure 4.3*** *A. niger transformation plates to study how the length of flanking sequences effect on the deletion and replacement success and efficacy. The shorter the flanking regions were, less there were transformants on selection plates.*

***Table 4.2*** *The number of colonies with correct replacements on the transformation plates when gaaX was deleted simultaneously replaced by pyrG using donor DNA cassettes with different size flanking sequences.*

| Flanking sequences (bp) | RNP (µl) | Colonies | Screened | Correct deletion of *gaaX* |
|---|---|---|---|---|
| 1500 | 7 | 24 | 6 | 4/6 |
| 1500 | 5 | 26 | 5 | 5/5 |
| 1500 | 0 | 12 (4 plates) | 4 | 0/4 |
| 1000 | 7 | 26 | 5 | 4/5 |
| 1000 | 5 | 12 | 8 | 8/8 |
| 1000 | 0 | 3 | 2 | 0/2 |
| 500 | 7 | 6 | 6 | 3/6 |
| 500 | 5 | 10 | 4 | 1/4 |
| 500 | 0 | 0 | 0 | - |
| 100 | 7 | 12 | 4 | 0/4 |
| 100 | 5 | 5 | 1 | 0/1 |
| 100 | 0 | 1 | 1 | 0/1 |

Since the method had worked extremely well, it was repeated with the target genomic site of *gaaA-gaaC*$_{0\text{-}264}$ to ensure that the functionality of the method was not related to a single gene. The transformation resulted in even more colonies on transformation plates, and all screened colonies had the correct *gaaA-gaaC*$_{0\text{-}264}$ bp replacement as seen in Table 4.3. Therefore, the replacement of some genes is easier than others, but the method's accuracy was equally 100%. With the designed gRNAs, the method's accuracy was not affected when crRNA was changed or two crRNAs were used. The method developed in this study is presented in Figure 11.

***Table 4.3*** *When gaaA:gaaC*$_{0\text{-}264}$ *was deleted, all screened colonies had correct gene deletion and replacement of gaaA:gaaC*$_{0\text{-}264}$ *by UDH and pyrG.*

| Deletion | crRNA | screened | ***gaaA:gaaC***$_{0\text{-}264}$ |
|---|---|---|---|
| *gaaA/C* | gERA-015 | 2 | 2/2 |
| *gaaA/C* | gERA-016 | 5 | 5/5 |
| *gaaA/C* | gERA-015 and -016 | 5 | 5/5 |
| *gaaA/C* | None (control) | 2 | 0/2 |

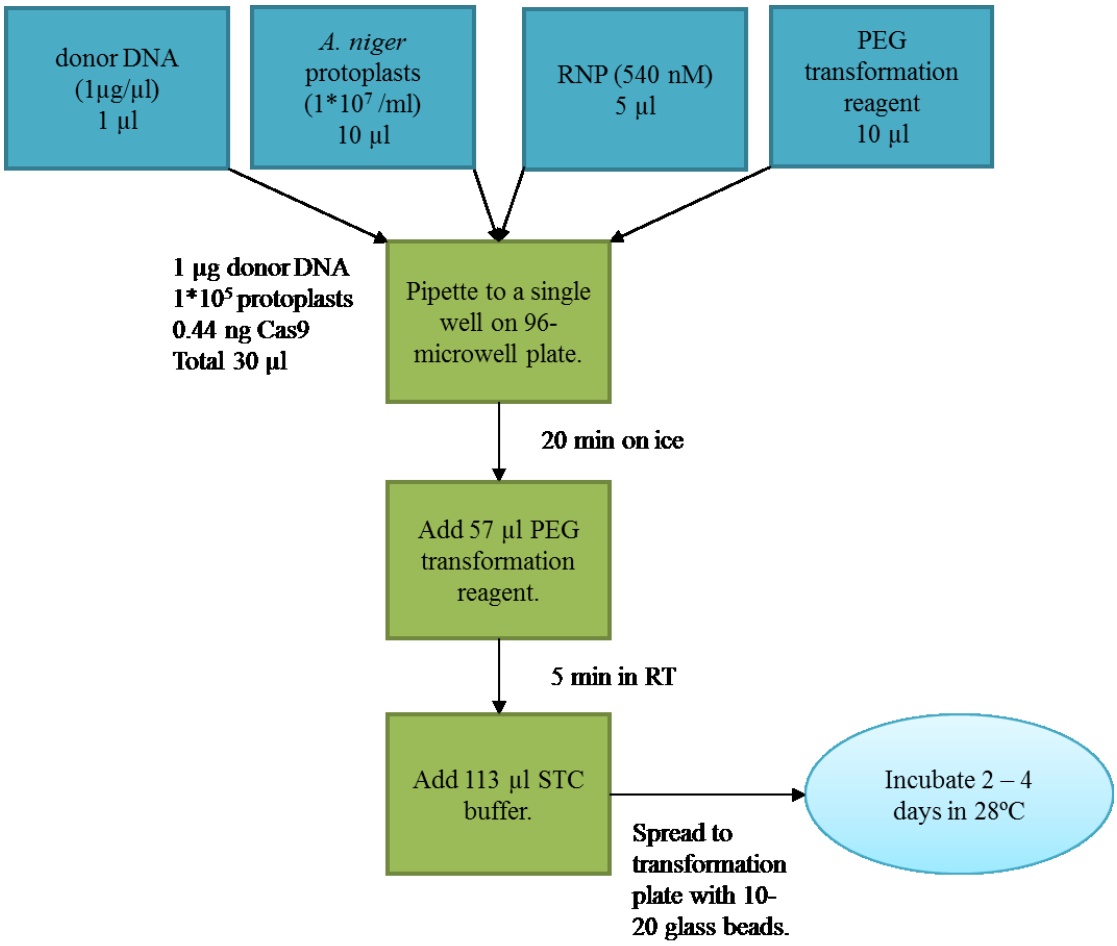



***Figure 4.4*** *The new, simple, and efficient CRISPR/Cas9 method for A. niger.*

### 4.1.4 Multiplexing

The new CRISPR/Cas9 protocol was tested to delete multiple genes, *gaaA*-*gaaC*$_{0\text{-}264\ bp}$, *39114* and *gaaX*, at the same time from Δ*pyrG* strain of *A. niger* and replace them by heterologous genes *UDH* and *pyrG* as seen in Figure 4.4. As seen from Table 4.4 and Table 4.5, simultaneous replacement of 2 or 3 genes succeeded, although not with high efficiency. When deleting 2 genes, 2 colonies out of 19 screened colonies had both correct deletions (10.5 %). As deleting 3 genes, 1 colony out of 16 screened colonies had all 3 correct deletions (6.3 %). Several colonies had only one correct deletion, since all donorDNA cassettes had same *pyrG* as a selection marker, and thus one correct replacement guaranteed survival on SCD-URA plates already. Control plates were quite empty as suspected and did not have any correct deletions.

Deletions were made with different combinations of gRNAs. Tables 4.4. and 4.5 confirm that gRNAs effect how easily gene is deleted. Also comparing the deletions of *gaaX* and *39114*, it seems that some genes are more easily deleted than others are. As a conclusion,

it may be better to delete a gene with two gRNAs while deleting or replacing multiple genes.

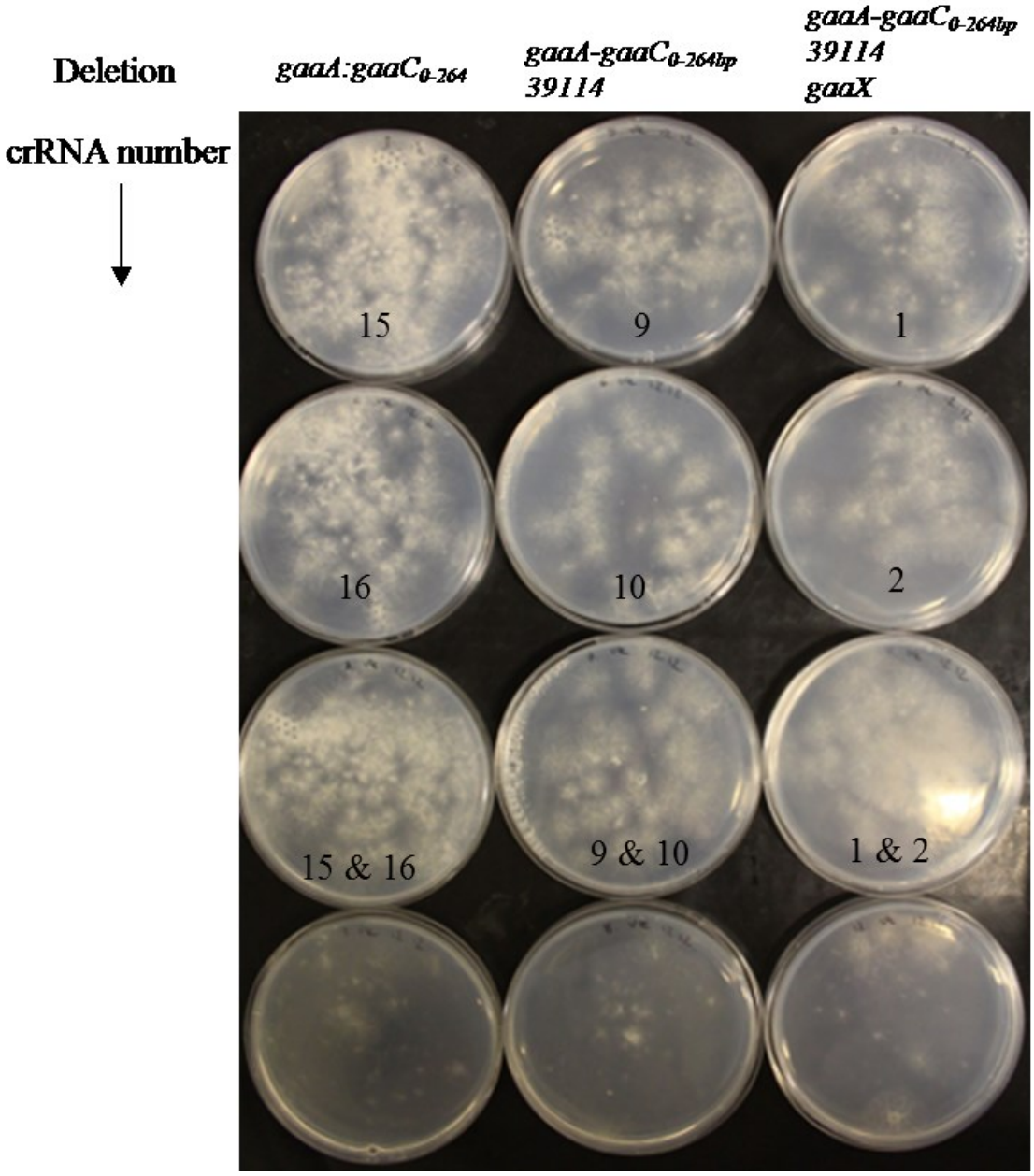



***Figure 4.5*** *The transformation plates using the developed CRIPRS/Cas9 method when one or more of the genes gaaA-gaaC$_{0\text{-}264bp}$, 39114, and gaaX were to be replaced by UDH and pyrG. The numbers on plates indicate the number of the crRNA that was used in RNP.*

***Table 4.4*** *The number of colonies with correct deletions and replacements when gaaA-gaaC$_{0\text{-}264\ bp}$ and 39114 were to be deleted simultaneously.*

| Deletion | crRNA | screened | Δ*gaaA-gaaC*$_{0\text{-}264}$ bp | Δ*39114* | 1 deletion | 2 deletions |
|---|---|---|---|---|---|---|
| *gaaA:gaaC*$_{0\text{-}264}$ bp, *39114* | gERA-009 and -015 | 4 | 3/4 | 1/4 | 2/4 | 1/4 |
| *gaaA:gaaC*$_{0\text{-}264}$ bp, *39114* | gERA-010 and -016 | 7 | 1/7 | 2/7 | 3/7 | 0/7 |
| *gaaA:gaaC*$_{0\text{-}264}$ bp, *39114* | gERA-009, -010,-015, and -016 | 8 | 1/8 | 3/8 | 2/8 | 1/8 |
| *gaaA:gaaC*$_{0\text{-}264}$ bp, *39114* | All together | 19 | 5/19 | 6/19 | 7/19 | 2/19 |
| *gaaA:gaaC*$_{0\text{-}264}$ bp, *39114* | None (control) | 2 | 0 | 0 | 0 | 0 |

***Table 4.5*** *The number of colonies with correct deletions and replacements when gaaA-gaaC$_{0\text{-}264\ bp}$, 39114 and gaaX were to be deleted simultaneously.*

| Deletion | gRNA | screened | Δ*gaaA-gaaC*$_{0\text{-}264}$ bp | Δ*39114* | Δ*gaaX* | 1 deletion | 2 deletions | 3 deletions |
|---|---|---|---|---|---|---|---|---|
| *gaaA:gaaC*$_{0\text{-}264}$ bp, *39114, gaaX* | gERA-001, -009, and -015 | 6 | 3/6 | 1/6 | 2/6 | 3/6 | 0/6 | 0/6 |
| *gaaA:gaaC*$_{0\text{-}264}$ bp, *39114, gaaX* | gERA-002,-010, and-016 | 5 | 1/6 | 1/6 | 4/6 | 2/6 | 2/6 | 1/6 |
| *gaaA:gaaC*$_{0\text{-}264}$ bp, *39114, gaaX* | gERA-001, -002, -009, -010, -015, and -016 | 5 | 2/5 | 2/5 | 2/5 | 2/5 | 2/5 | 0/5 |
| *gaaA:gaaC*$_{0\text{-}264}$ bp, *39114, gaaX* | All together | 16 | 6/16 | 4/16 | 8/16 | 7/16 | 4/16 | 1/16 |
| *gaaA:gaaC*$_{0\text{-}264}$bp, *39114, gaaX* | None (control) | 2 | 0 | 0 | 0 | 0 | 0 | 0 |

## 4.2 Mucic acid production

When the first gene, *gaaX*, was deleted with optimized CRISPR/Cas9 method from Δ*pyrG A. niger*, it's phenotype was verified to have effect on the pectin catabolizing enzymes. Preliminary results from this enzyme assay proved that galacturonic acid reductase, *gaaA*, was around 4 times more active in Δ*gaaX* than in WT. As expected, GaaX effects on the production of proteins that are related to pectin catabolism. Study was continued with combining multiple deletions to produce MA by *A. niger* cultivations. After new strains were generated, they were cultivated and compared with old strains.

In all 3 different media, the biomass of old strains accumulated more compared to new strains. Especially, the unique *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ Δ*39114* Δ*gaaX UDH* strain produced the least biomass. Thus, partial disruption of *gaaC*, the transcription of *UDH* under *gaaB* promoter instead of *gpda* promoter and the deletion of *gaaX* influenced the growth of the strains: new strains did not use GA for growth as much as the old strains. In Figure 4.5, all strains have grown on YP+GA, YP+GA+Glu or YP+Pectin for 2 days as 3 replicates. By deleting *39114*, the colour of cultivations turned browner in all media.

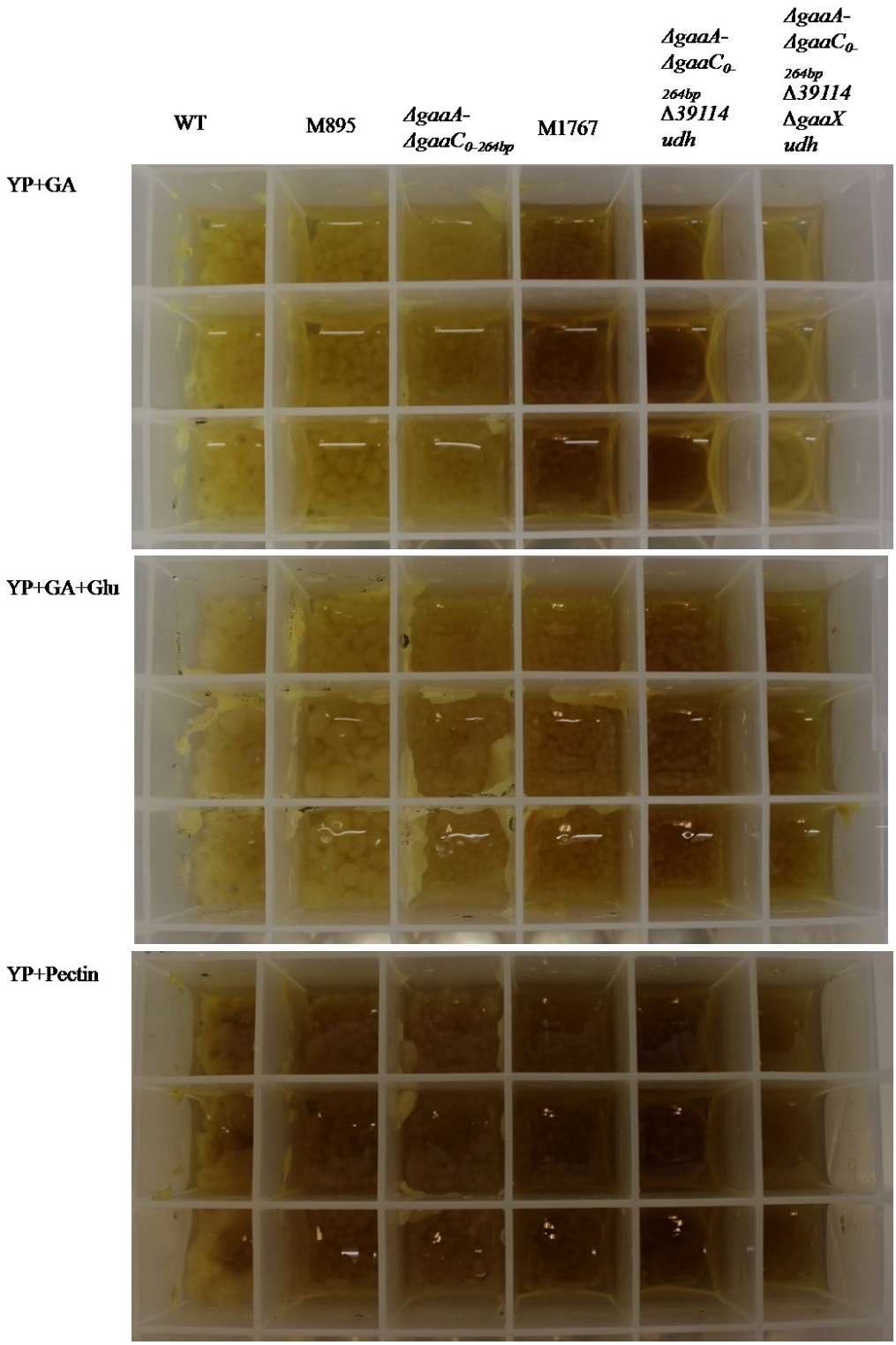



***Figure 4.6*** *Three parallel cultivations (4 ml) of, WT, old strains M895 and M1767 and engineered A. niger strains in YP+GA, YP+GA+Glu and YP+Pectin after two days. WT produced the most biomass as expectedly. The more the strain had deletions less it produced biomass. New strains grew better on YP+GA+Glu and YP+Pectin than on YP+GA since they contained more compounds for growth.*

Figures 4.6, 4.7, and 4.8 include HPLC results for mucic acid concentrations from different cultivations after 24, 48 and 120 hours. Each result is an average between three parallel cultivations and the standard deviation can be seen in the graph if the standard deviation is bigger than the strain's unique symbol. As expected for the negative control, wild type strain of *A. niger* used all GA and pectin without production of MA since *gaaA* was present. As expected, Δ*gaaA* strains, without *39114* deletion, did not produce much of MA, since *39114* consumes galactaric acid. However, some MA could be seen in new Δ*gaaA UDH* cultivations in YP+GA and YP+Pectin.

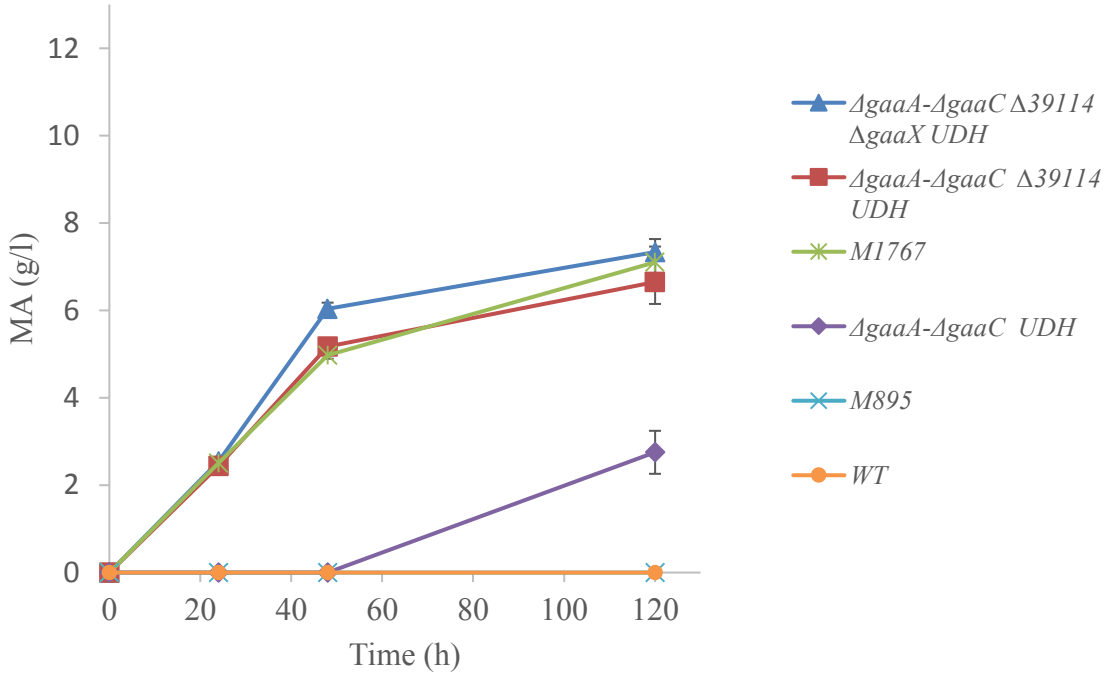



***Figure 4.7*** *The MA production in YP+GA*

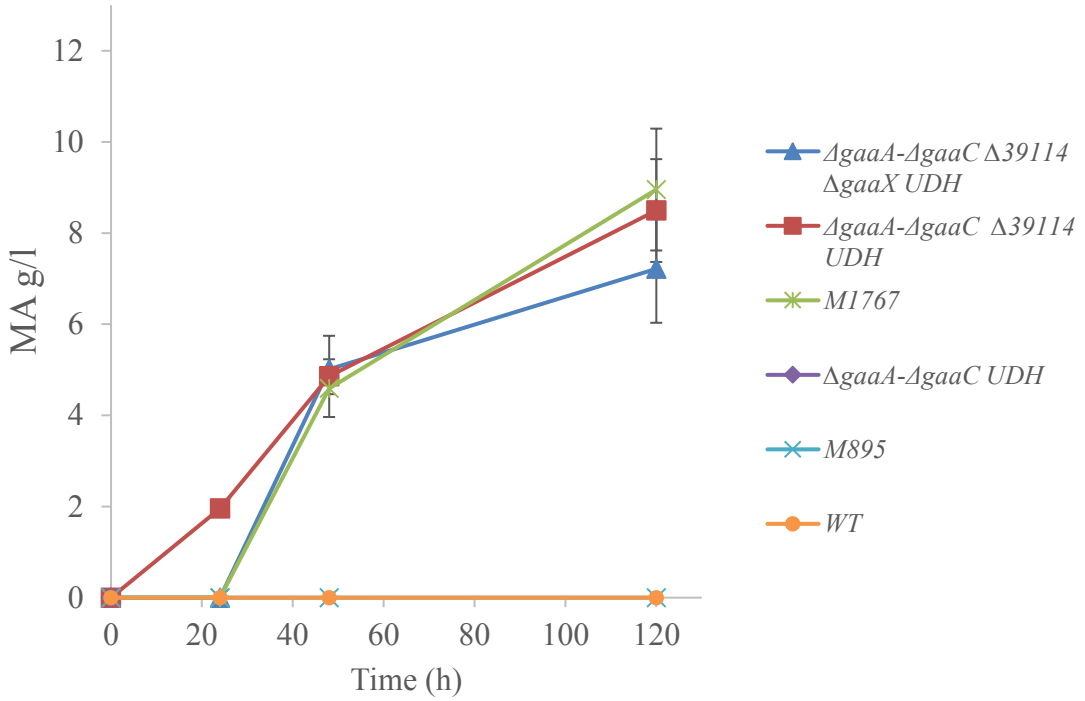



***Figure 4.8*** *The MA production in YP+GA+Glu*

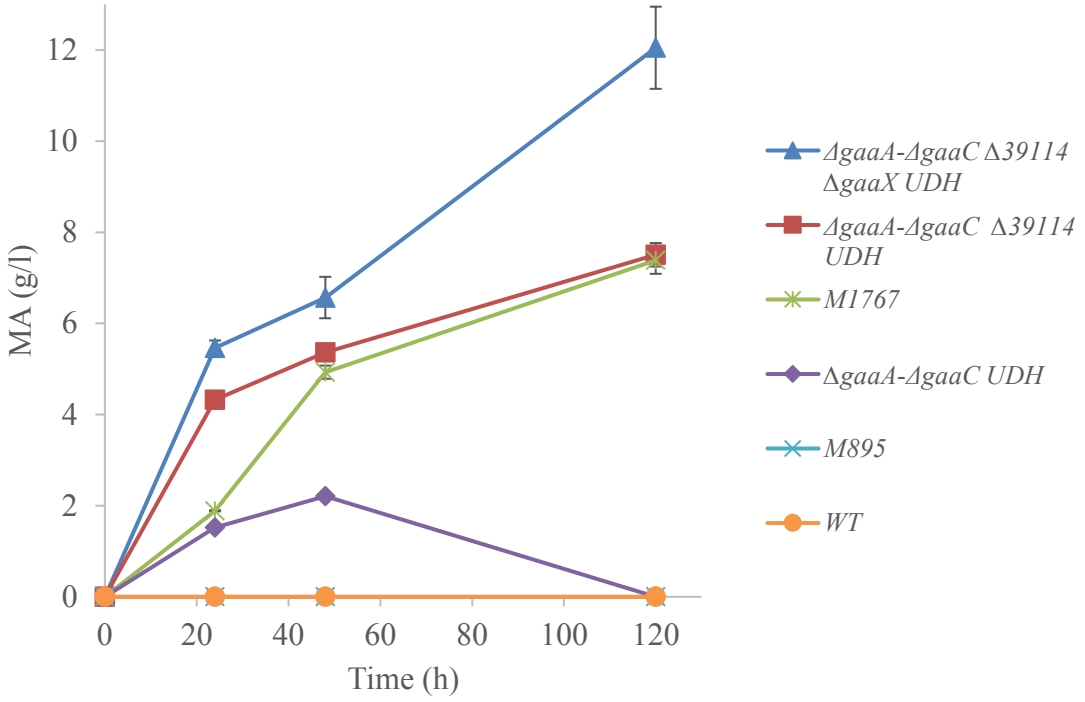



***Figure 4.9*** *The MA production in YP+Pectin*

According to Figure 4.6 and Figure 4.7, when grown in YP+GA or YP+GA+Glu medium, strains with deletion of *39114* and *gaaX* produced MA with only minor differences to old strains. In YP+GA, *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ *Δ39114 ΔgaaX UDH* produced MA slightly

faster between 24 and 48 hours than Δ*gaaA* Δ*39114 UDH* strains. Altogether, *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ Δ*39114* Δ*gaaX UDH* produced MA 7.33 ± 0.13 g/l after 5 days. As for other strains, Δ*gaaA* Δ*39114 UDH* produced 6.65 ± 0.5 g/l while M1767 produced 7.10 ± 0.53 g/l of mucic acid. When glucose was present, the strains acted quite similarly but the order in MA productivity shifted, as seen in Figure 4.8. M1767 cultivation contained MA 8.95 ± 1.34 g/l, *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ Δ*39114 UDH* 8.49 ± 1.13 g/l and *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ Δ*39114* Δ*gaaX UDH* 7.22 ± 1.18 g/l after 5 days. The standard deviations are probably higher because the cells grew faster with glucose and the cultivations started to have a lot of biomass compared to the liquid in the solution. As a result, identical samples were hard to generate.

As expected, major differences appeared between YP+Pectin cultivations as seen in Figure 4.9. *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ Δ*39114* Δ*gaaX UDH* produced 12.05 ± 0.9 g/l of MA in 5 days while *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ Δ*39114 UDH* produced only 7.51 ± 0.26 g/l of MA and M1767 produced 7.38 ± 0.30 g/l of MA. This confirms that *gaaX* deletion results immediately to an increased amount of free GaaR and the new strain produced more proteins related to pectin catabolism. Up to 48 hours, *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ Δ*39114 UDH* produced MA mucic acid faster than M1767, but its productivity slowed down and strains resulted in the almost same concentrations of MA.

Figures 4.9, 4.10 and 4.11 indicate the consumption of galacturonic acid in three different cultivations. Unfortunately, the exact consumption cannot be seen in Δ*39114* strains due to evaporation in 4 ml cultivations of in YP+GA and YP+GA+Glu. When GA concentration decreased only a bit and water evaporated regrettably a considerate amount, the concentration only increased, leading to high standard deviations. Still, the order of consumption reflects the results of MA production in strains *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ Δ*39114* Δ*gaaX UDH*, *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ Δ*39114 UDH* and M1767. Strains M895, *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ *UDH* and WT used more GA since they could catabolize it further than MA, finally into energy. As a result, their consumption of GA is observable. Interestingly, *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ strain uses GA faster than the old strain of M895 with the same deletion and *UDH.*

In Figure 4.11 strains produce GA when pectin is catabolized into smaller molecules and finally into GA. At the same time, GA is converted to MA or catabolized into energy. As in YP+GA or YP+GA+Glu, M895, *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ *UDH* and WT consumed GA much faster than Δ*39114* strains, since GA is led to energy production. Consequently, these strains also grow faster in biomass than Δ*39114* strains in which GA content only increases and is used for MA production during five days. After 5 days, *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ Δ*39114* Δ*gaaX UDH* contained 10.63 ± 0.13 g/l, *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ Δ*39114 UDH* 11.73 ± 0.22 g/l and M1767 13.06 ± 0.39 g/l of GA.

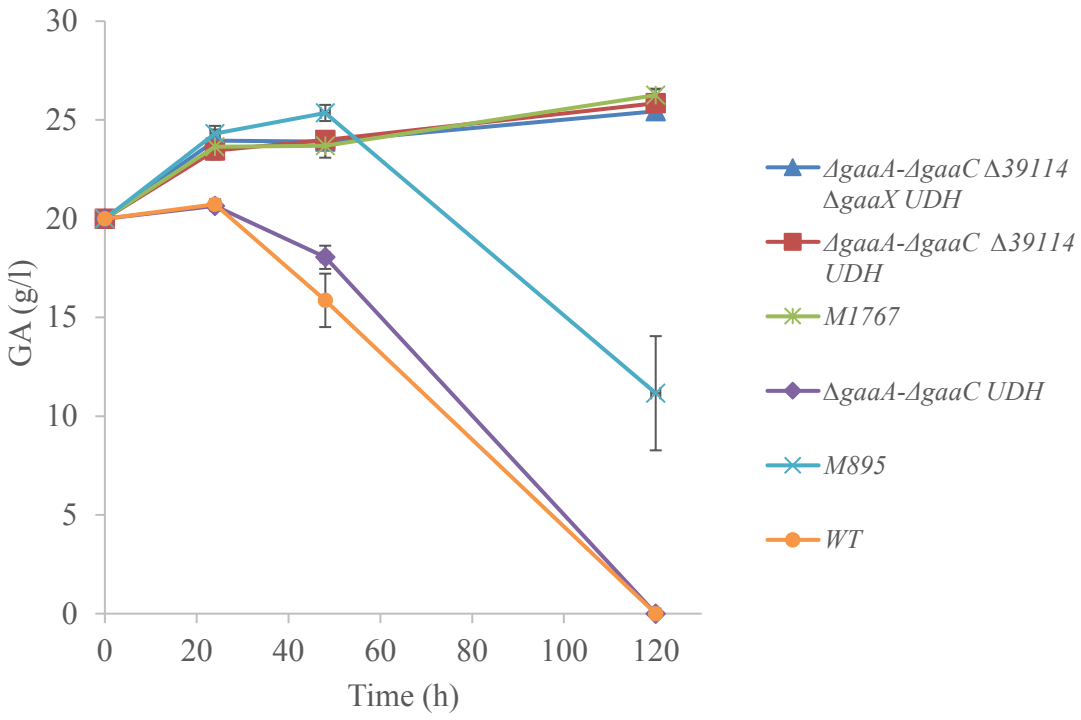



***Figure 4.10*** *The GA consumption in YP+GA*

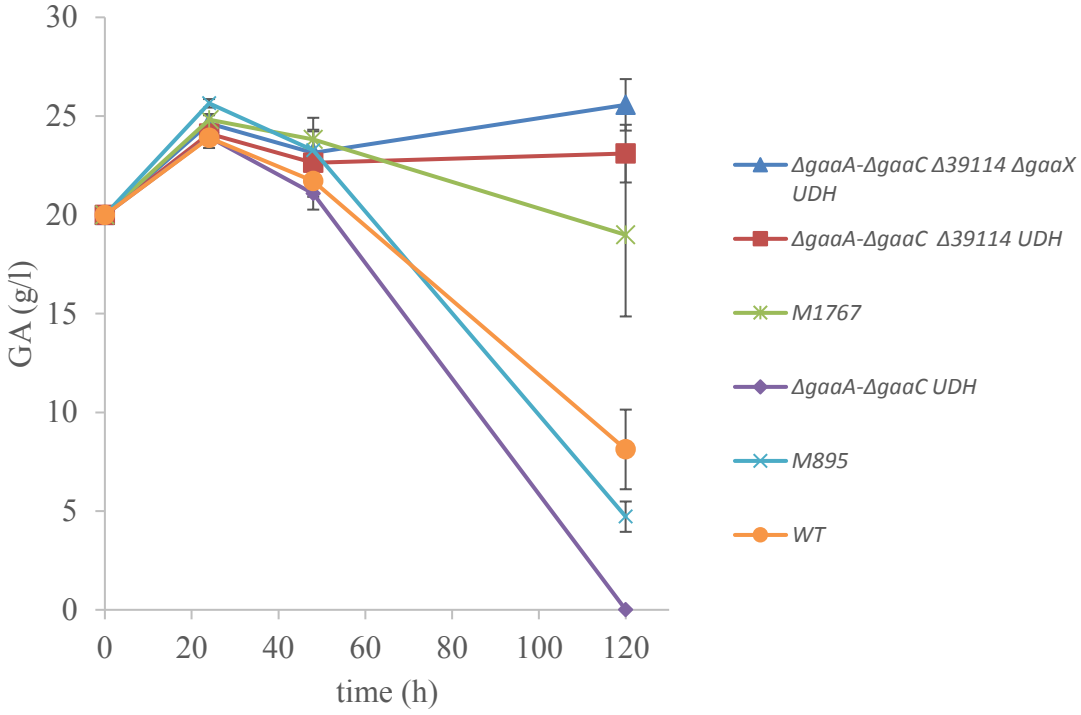



***Figure 4.11*** *The GA consumption in YP+GA+Glu*

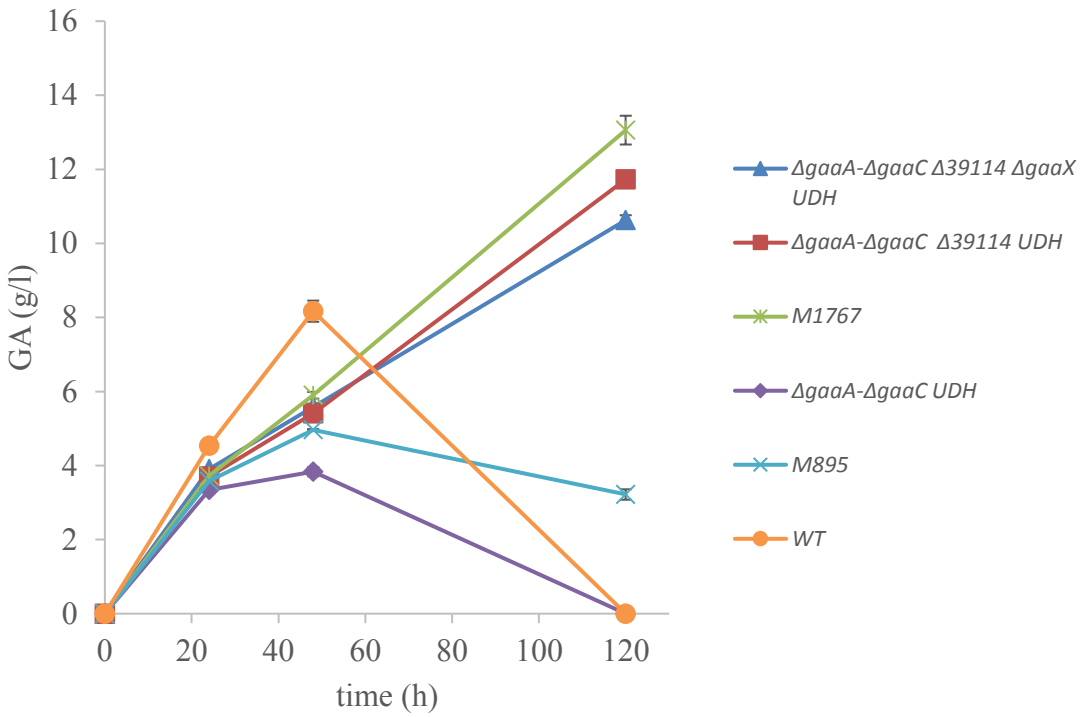



***Figure 4.12*** *The GA formation and consumption in YP+Pectin*

# 5. DISCUSSION

In this study, the use of CRISPR/Cas9 genome editing method based on *in vitro* assembled RNPs was reported for the first time in *A. niger*. In addition, the reaction volumes in the method were downscaled enabling the use of 96-microwell plates and high-throughput workflows, which can be facilitated by the use of liquid handling robots. The method is simple, accurate, and efficient, and it could be tried in other filamentous fungi, for example in industrially relevant *A. oryzae*. The amount of RNP-complex effects the number of colonies and correct replacements by CRISPR/Cas9 technology. When 5 µl of RNP-mix was used instead of 7 µl or 9 µl, there were more colonies growing on the transformation plates and more correct deletions amongst these colonies. This effect was observed with a donor DNA cassette with 1500 bp long flanking sequences. The RNP-mix may have been toxic to *A. niger* in bigger concentrations since Cas9 has been harmful to other cells as well (Korppoo 2017). The large amount of RNP-mix may have also affected to excess, repaired DSB by NHEJ and caused off-target mutations, since there are several colonies with incorrect replacements when using 7 µl or 9 µl of RNP.

The length of flanking sequences in the donor DNA cassettes affected as well to the number of colonies and correct gene replacements. When flanking sequences of 1500 bp or 1000 bp were applied to replace a single gene, all transformants had the correct replacement. The donor DNA cassette with 500 bp long flanking sequences was still sufficient to give some correct replacements but with 100 bp long flanking sequences there were not any. Most likely NHEJ pathway has functioned instead of HDR with shorter flanking sequences when the unexpected results occurred but the colonies still appeared on selective plates. Thus, *A. niger* needs longer flanking sequences to have absolutely correct HDR functioning since NHEJ is the predominant way of repairing double strand breaks in *A. niger*.

The combination of the different size in flanking sequences and different amount of RNP-mix effected the number of colonies and correct deletions in these colonies. It seems that with shorter flanking sequences 7 µl of RNP-mix gave more correct deletions than with 5 µl of RNP-mix and so 2 µl might have been boosting up the homologous recombination. All in all the combination of 5 µl of RNP-mix to 1000 or 1500 bp long flanking sequences in the donor DNA cassette gave the best results.

When $1*10^6$ *A. niger* protoplasts were used for one transformation in CRISPR/Cas9 method, the control plates were also full of colonies. Consequently, the more there were protoplasts, the more there were colonies in which the donor DNA cassette had integrated randomly into the genome as seen in control plates. After diluting the protoplast solution and applying only $1*10^6$ protoplast per transformation, control plates had only few colonies. There were enough of colonies in the transformation plates even though

CRISPR/Cas9 procedure was downscaled to plating 200 µl of the final solution. Thus, the CRISPR/Cas9 deletion procedure was tried with a robot by Sami Holmström and Joosu Kuivanen. The method did not give as good results as doing the procedure by hand. Failed pipetting to the bottom of the wells or missing mixing of the reagents may have probably caused lower success rate when using robotics.

The colonies growing on the selective plates without correct deletions by homologous recombination might have been due to several reasons. If the deletion target was still observed, the donor DNA cassette had gone somewhere else in genome by NHEJ giving the ability to survive the selection. Some unexplained bands appeared to incorrect deletion strains as well. Herein, the donor DNA cassette might have integrated to the cut site by NHEJ, might have even turned around in the process or integrated only partly. Homologous recombination had worked only half way, when only 1$^{st}$ or 2$^{nd}$ PCR gave the correct PCR product. The unfortunate phenomenon was occurrence of colonies that seemed to have correct deletion but also still had the target gene. In other words, these colonies had integrated nucleus or somehow two copies of the same gene or the gene had been deleted but also inserted back to some other locus in the genomes. Another possibility is that the colonies were mixed due to unknown reasons although single colonies were picked and purified through multiple cultivations.

With CRISPR/Cas9 system based on RNP transformation, laborious cloning steps were avoided when Cas9 and gRNA expression were left out. Although, donor DNA cassettes had to be constructed. Fortunately, MoClo system helped cloning, and system eases always after the first cloning procedure when there are ready entry vectors to work with. Limitation in the MoClo are the unwanted extra BsaI, BsmBI and NotI sites in the sequences to be cloned, as in *gaaA* deletion 264 bp of the next gene had to also be deleted to gain more than 1000 bp flanking sequence upstream of the *gaaA*.

For future, there exists different ways to avoid extra cloning steps. Herein 100 bp flanking sequences did not work when NHEJ pathway was active. An interesting opportunity would be to disrupt NHEJ pathways and afterwards try shorter flanking sequences. This would reduce cloning steps when only one PCR could be used to make donor DNA cassette, but would also require a stable strain with disrupted NHEJ, which may be impossible. In addition, an attractive opportunity would be to try two gRNA method targeting the start and end sequences of the gene with donor DNA cassettes that had 50 bp flanking sequences, like in *A. fumigatus* (Al Abdallah *et al.* 2017). Thirdly, since the gene disruption has been succeeded in *A. oryzae* with linear plasmids with selection markers without any flanking sequences through NHEJ (Zheng *et al.* 2017), this could be tried and use the same linear donor DNA for several deletions. If the selective markers were to be avoided, one option would be to use supplementary AMA1 plasmid during transformation to indicate successful transformation.

Since the selection marker was same in all different donor DNA cassettes, it was only natural that colonies could survive with only one correct gene replacement while multiplexing. This is most likely the reason why most of the colonies had only one deletion. Overall, multiplexing worked well, considering the fact that all targets had same selective marker. Since the method worked with one selection marker, the method would function probably even better with different selection markers for different genes to be multiplexed. Multiplexing has been only done once before for *A. niger* with a plasmid expressing different gRNAs, while small modifications and one insertion were made, but no gene replacements as in this study (Nødvig *et al.* 2018).

The new strain *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ Δ*39114* Δ*gaaX UDH* produced mucic acid faster in YP+GA than the double mutation strains. This is most likely due to three active *UDH* genes instead of 2 or increased GA transporter production due to *gaaX*. When old double mutant M1767 and *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ Δ*39114 UDH* are compared, it seems *gaaB* promoter works as well as the *gpdA* promoter in inductive conditions with GA.

In pectin cultivations, as expected, triple mutant produced more MA than double mutant since GaaX is not repressing GaaR activation of genes at all in pectin degrading network. GA is produced faster and thus it is converted into MA faster. Overall, MA was produced more in pectin cultivations than with YP+GA or YP+GA+GLU. This phenomenon is most likely to due to extra energy for cells and transporters and other molecules gained from pectin. The function mechanisms of GA transporters is still to be characterized: if the transporters need ATP to work, then the additional substrates enhance their activity. If they work through diffusion, then additional substrates have no effect on them.

The effect of the disruption of *gaaC,* in other words the accumulation of inductive molecule, was seen between the new and old double and single mutants. New *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ Δ*39114 UDH* produces MA faster than M1767 because of the induction of pectin related enzymes. Also *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ *UDH* produced more MA than M895 in inductive conditions, which backs up the conclusion that gaaC disruption caused considerate positive inductive impact. Disruption of *gaaC* may have also affected the triple mutant since still a little amount of 2-keto-3-deoxy-galactonate is formed because *gaaA* activity can be partly replaced as explained in chapter 2.1.3.

As for future prospects, the new strain could be cultivated on real citrus peel or sugar beet pulp for MA production testing. The fermentation conditions could be optimized in terms of temperature, pH, additional substrates and humidity, and the whole process could be scaled up to get even faster MA production. As *A. niger* produces already more MA in 4 ml than T. reesei and produces its own pectinases, *A. niger* fermentation of pectin-rich materials into MA is most likely more applicable to industrial use than *T. reesei*. The same metabolic engineering steps could also be done to similarly industrially relevant, pectinase-degrading *A. oryzae*, since donor DNA cassettes were constructed for *A. oryzae* genes homologous to *A. niger* ones. Also further metabolic engineering could fasten up

the MA production. For example, the general repressor *creA* could be deleted, but might have unexpected results in other metabolism, or *gaaR* activator gene moved under constitutive promoter.

# 6. CONCLUSIONS

To the best of my knowledge, this is the first published study for *A. niger* about a successful CRIPSR/Cas9 genome editing method by transformation of *in vitro* assembled RNP composed of a synthetic gRNA and Cas9. Three genes, *gaaA*, *39114* and *gaaX* were replaced by *pyrG* and *UDH* through homology directed repair and introduction of donor DNA cassettes. In addition, *gaaC,* a gene next to *gaaA*, was disrupted by replacement of the first 264 bp of the gene, while *gaaA* was replaced. Notably, this is also the first time that genes have been replaced simultaneously in *A. niger*, and the first time of multiplexing with RNP method in filamentous fungi.

The new CRISPR/Cas9 method was first confirmed to function in *A. niger*, after which the method was optimized and downscaled to fit 200 µl scale 96-microwell plate. This makes the method suitable to be carried out with a liquid-handling robot. 5 µl of 540 nM RNP-mix (0.44 ng Cas9) was the best amount to gain correct replacements through HDR and PEG-transformation. With 1*10^5 protoplasts per transformation, control plates contained only a few colonies decreasing the background to a minimum. Finally with flanking sequences between 1000 bp and 1500 bp worked the best in gene replacement in *A. niger* without disrupted NHEJ pathway. Finally, one gene replacement could be done in a downscaled format with 100% frequency of correct colonies on transformation plates.

Genes were replaced simultaneously by using the donor DNA cassettes that had the same selection marker for all genes. One correct triple mutant with three gene replacements was found from a group of 16 screened colonies (6.25 %), while targeting 3 genes. When targeting 2 genes, 2 correct double mutants was found within 19 screened colonies (10.53 %). All though the success rate is not that high, it does not require multiple laborious consecutive deletions. Of course, multiplexing could work better if different markers were used for different deletions.

All in all, new efficient, accurate, and simple CRISPR/Cas9 protocol was developed in this study, and it can be used for future deletions, insertions, modifications and replacements in *A. niger*. It could be tried for other filamentous fungi as well. Deletion cassettes for HDR were constructed through recently published modular cloning system for *S. cereviase*. MoClo works for genes in *A. niger* and *A. oryzae* as well, although possible disturbing BsaI, BsmBI and NotI sites should be checked before cloning.

As expected, the new strain *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ *Δ39114 ΔgaaX UDH* degraded citrus pectin more efficiently than the strain without *gaaX* deletion, and converted it faster into mucic acid than *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ *Δ39114 UDH* or old strain *ΔgaaA Δ39114 UDH*. While grown on GA, triple mutant strain produced MA faster than other strains, probably

due to its three copies of *UDH* instead of two. When new and old double mutants were grown on GA, no significant difference could be seen between MA productions. So the GA-responsive promoter of *gaaB* worked as well as the constitutive promoter in *UDH* expression. On the other hand, the effect of *gaaC* disruption, resulting in the accumulation of the inductive molecule, could be seen between the old and new double mutant strains. Δ*gaaC* resulted in faster pectin degradation, since new strain produced faster MA than the old one during the first 48 hours.

*ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ Δ*39114* Δ*gaaX UDH* produces 12.05 g/l mucic acid from cultivation with 20 g/l citrus pectin in 4 ml in 5 days. Whereas, the old strain, *ΔgaaA* Δ*39114 UDH* produces MA only 7.31 g/l in 5 days. *T. reesei* produces in mucic acid concentration of 10.5 g/l from 20 g/l prehydrolyzed pectin in 4 ml 5 days lasting cultivation (Paasikallio *et al.* 2017). As a conclusion, *A. niger* already competes with T. reesei in efficient mucic acid production. Moreover, *A. niger* produces its own pectinases for pectin degradation while T. reesei does not. In the future, optimization and upscaling of the *ΔgaaA-ΔgaaC*$_{0\text{-}264bp}$ Δ*39114* Δ*gaaX UDH* cultivation can increase MA productivity even more.

# REFERENCES

Al Abdallah, Q., Ge, W. and Fortwendel, J. R. (2017) 'A Simple and Universal System for Gene Manipulation in Aspergillus fumigatus: In Vitro-Assembled Cas9-Guide RNA Ribonucleoproteins Coupled with Microhomology Repair Templates.', *mSphere*, 2(6),

Acree Salomon Farley (1920) 'Method of converting wood into mucic acid'.

Aittomäki, E., Leisola, M. and Weymarn, N. von (2002) *Bioprosessitekniikka*. WSOY.

Alazi, E., Khosravi, C., Homan, T., du Pré, S., Arentshorst, M., Di Falco, M., Pham, T., Peng, M., Aguilar-Pontes, M., Visser, J., Tsang, A., de Vries, R. and Ram, A. (2017) 'The pathway intermediate 2-keto-3-deoxy-L-galactonate mediates the induction of genes involved in D-galacturonic acid release and catabolism', *submitted to FEBS Letters*, 591, pp. 1408–1418.

Alazi, E., Knetsch, T., Falco, M. Di, Reid, I. D., Arentshorst, M., Visser, J., Tsang, A. and Ram, A. F. J. (2018) 'Inducer-independent production of pectinases in Aspergillus niger by overexpression of the Dgalacturonic acid-responsive transcription factor gaaR', *Applied Microbiology and Biotechnology*. Applied Microbiology and Biotechnology, 10, pp. 2723–2736.

Anonymous (1922) 'MucicAcid-1920s', *Chemical and Metallurgical Engineering*.

Barrangou, R. and Marraffini, L. A. (2014) 'CRISPR-cas systems: Prokaryotes upgrade to adaptive immunity', *Molecular Cell*. Elsevier Inc., 54(2), pp. 234–244.

Barth, D. and Wiebe, M. G. (2017) 'Enhancing fungal production of galactaric acid', *Applied Microbiology and Biotechnology*. Applied Microbiology and Biotechnology, 101, pp. 4033–4040.

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., Marraffini, L. A. and Zhang, F. (2010) 'Multiplex Genome Engineering Using CRISPR/Cas Systems', *Science*, 339(February), pp. 819–823.

Deng, H., Gao, R., Liao, X. and Cai, Y. (2017) 'CRISPR system in filamentous fungi: Current achievements and future directions', *Gene*. Elsevier, 627(4), pp. 212–221.

Doran-Peterson, J., Cook, D. M. and Brandon, S. K. (2008) 'Microbial conversion of sugars from plant biomass to lactic acid or ethanol', *Plant Journal*, 54(4), pp. 582–592.

Doran, J. B., Cripe, J., Sutton, M. and Foster, B. (2000) 'Fermentations of Pectin-Rich Biomass with Recombinant Bacteria to Produce Fuel Ethanol', *Applied Biochemistry and Biotechnology*, 84–86(1–9), pp. 141–152.

Engler, C., Kandzia, R. and Marillonnet, S. (2008) 'A one pot, one step, precision cloning method with high throughput capability', *PLoS ONE*, 3(11).

Graaff, L. De, Broek, H. Van Den and Visser, J. (1988) 'Isolation and Transformation

ofthe *pyrG*uvate kinase gene of Aspergillus nidulans', *Current Genetics*, 13, pp. 315–321.

Grahl, N., Demers, E. G., Crocker, A. W. and Hogan, D. A. (2017) 'Use of RNA-Protein Complexes for Genome Editing in Non-albicans Candida Species', *mSphere*, 2(3),

Hsu, P. D., Lander, E. S. and Zhang, F. (2014) 'Development and applications of CRISPR-Cas9 for genome engineering', *Cell*. Elsevier, 157(6), pp. 1262–1278.

de Jong, E., Dam, M. A., Sipos, L. and Gruter, G.-J. M. (2012) 'Furandicarboxylic Acid (FDCA), A Versatile Building Block for a Very Interesting Class of Polyesters', in *Biobased monomers, polymers and materials*, pp. 1–13.

Katayama, T., Tanaka, Y., Okabe, T., Nakamura, H., Fujii, W., Kitamoto, K. and Maruyama, J. ichi (2016) 'Development of a genome editing technique using the CRISPR/Cas9 system in the industrial filamentous fungus Aspergillus *oryzae*', *Biotechnology Letters*. Springer Netherlands, 38(4), pp. 637–642.

Kaya, M., Sousa, A. G., Crépeau, M. J., Sørensen, S. O. and Ralet, M. C. (2014) 'Characterization of citrus pectin samples extracted under different conditions: Influence of acid type and pH of extraction', *Annals of Botany*, 114(6), pp. 1319–1326. Keasling, J. D. (2003) 'Manufacturing Molecules Through Metabolic Engineering', *Science*, 24(1–2), pp. 1355–1358.

Kiely, D. A. and Hash, K. R. (2007) 'Method of oxidation using nitric acid'.

Kim, D., Bae, S., Park, J., Kim, E., Kim, S., Yu, H. R., Hwang, J., Kim, J.-I. and Kim, J.-S. (2015) 'Digenome-seq: genome-wide profiling of CRISPR-Cas9 off-target effects in human cells', *Nature Methods*, 12(3), pp. 237–243.

Knuf, C. and Nielsen, J. (2012) 'Aspergilli: Systems biology and industrial applications', *Biotechnology Journal*, 7(9), pp. 1147–1155. doi: 10.1002/biot.201200169.

Korppoo, A. (2017) *Development of the CRISPR / Cas9 Method for Use in T . reesei*.

Kowalczyk, J. E., Lubbers, R. J. M., Peng, M., Battaglia, E., Visser, J. and Vries, R. P. De (2017) 'Combinatorial control of gene expression in Aspergillus niger grown on sugar beet pectin', *Scientific Reports*. Springer US, 7(May), pp. 1–12.

Kuivanen, J., Dantas, H., Mojzita, D., Mallmann, E., Biz, A., Krieger, N., Mitchell, D. and Richard, P. (2014) 'Conversion of orange peel to L-galactonic acid in a consolidated process using engineered strains of Aspergillus niger', *AMB Express*, 4(1), pp. 1–8.

Kuivanen, J., Penttilä, M. and Richard, P. (2015) 'Metabolic engineering of the fungal D-galacturonate pathway for L-ascorbic acid production', *Microbial Cell Factories*, 14(1), pp. 1–9.

Kuivanen, J., Wang, Y.-M. J. and Richard, P. (2016) 'Engineering Aspergillus niger for galactaric acid production: elimination of galactaric acid catabolism by using RNA sequencing and CRISPR/Cas9', *Microbial Cell Factories*. BioMed Central, 15(1), p. 210.

Kuorelahti, S., Kalkkinen, N., Penttilä, M., Londesborough, J. and Richard, P. (2005)

'Identification in the mold Hypocrea jecorina of the first fungal D-galacturonic acid reductase', *Biochemistry*, 44(33), pp. 11234–11240.

Labun, K., Montague, T. G., Gagnon, J. A., Thyme, S. B. and Valen, E. (2016) 'CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering', *Nucleic acids research*, 44(W1), pp. W272–W276.

Lee, M. E., DeLoache, W. C., Cervantes, B. and Dueber, J. E. (2015) 'A Highly Characterized Yeast Toolkit for Modular, Multipart Assembly', *ACS Synthetic Biology*, 4(9), pp. 975–986.

Lewkowski, J. (2001) 'Convenient Synthesis of Furan-2,5,-dicarboxylic Acid and Its Derivates', *Polish journal of chemistry*, 75, pp. 1943–1946.

Li, X., Wu, D., Lu, T., Yi, G., Su, H. and Zhang, Y. (2014) 'Highly efficient chemical process to convert mucic acid into adipic acid and DFT studies of the mechanism of the rhenium-catalyzed deoxydehydration', *Angewandte Chemie - International Edition*, 53(16), pp. 4200–4204.

Liu, R., Chen, L., Jiang, Y., Zhou, Z. and Zou, G. (2015) 'Efficient genome editing in filamentous fungus Trichoderma reesei using the CRISPR/Cas9 system', *Cell Discovery*, 1, pp. 1–11.

Mable, B. K. and Otto, S. P. (1998) 'The evolution of life cycles with haploid and diploid phases', *BioEssays*, 20(6), pp. 453–462.

Mamma, D., Kourtoglou, E. and Christakopoulos, P. (2008) 'Fungal multienzyme production on industrial by-products of the citrus-processing industry', *Bioresource Technology*, 99(7), pp. 2373–2383.

Martens-uzunova, E. S. and Schaap, P. J. (2008) 'An evolutionary conserved D -galacturonic acid metabolic pathway operates across filamentous fungi capable of pectin degradation', *Fungal Genetics and Biology*. Elsevier Inc., 45(11), pp. 1449–1457.

Martens-Uzunova, E. S. and Schaap, P. J. (2009) 'Assessment of the pectin degrading enzyme network of Aspergillus niger by functional genomics.', *Fungal genetics and biology*. Elsevier Inc., 46 Suppl 1(1), pp. S170–S179.

McVey, M. and Lee, S. E. (2008) 'MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings', *Trends in Genetics*, 24(11), pp. 529–538.

Mojzita, D., Wiebe, M., Hilditch, S., Boer, H., Penttila, M. and Richard, P. (2010) 'Metabolic Engineering of Fungal Strains for Conversion of D Galacturonate to meso-Galactarate', *Applied and Environmental Microbiology*, 76(1), pp. 169–175.

Montague, T. G., Cruz, J. M., Gagnon, J. A., Church, G. M. and Valen, E. (2014) 'CHOPCHOP: A CRISPR/Cas9 and TALEN web tool for genome editing', *Nucleic Acids Research*, 42(W1), pp. 401–407.

NGA, B. H., Siew-Poh, T. and Lim, G. (1975) 'The Occurrence in Nature of a Diploid Strain of Aspergillus niger', *Journal of General Microbiologq*, 88, pp. 364–366.

Nielsen, J. and Keasling, J. D. (2016) 'Engineering Cellular Metabolism', *Cell*. Elsevier Ltd, 164(6), pp. 1185–1197.

Niu, J., Alazi, E., Reid, I. D., Arentshorst, M., Punt, P. J., Visser, J., Tsang, A. and Ram, A. F. J. (2017) 'An evolutionarily conserved transcriptional activator-repressor module controls expression of genes for D-Galacturonic acid utilization in Aspergillus niger', *Genetics*, 205(1), pp. 169–183.

Nødvig, C. S., Hoof, J. B., Kogle, M. E., Jarczynska, Z. D., Lehmbeck, J., Klitgaard, D. K. and Mortensen, U. H. (2018) 'Efficient oligo nucleotide mediated CRISPR-Cas9 gene editing in Aspergilli', *Fungal Genetics and Biology*, (January).

Nødvig, C. S., Nielsen, J. B., Kogle, M. E. and Mortensen, U. H. (2015) 'A CRISPR-Cas9 system for genetic engineering of filamentous fungi', *PLoS ONE*, 10(7), pp. 1–18.

Paasikallio, T., Huuskonen, A. and Wiebe, M. G. (2017) 'Scaling up and scaling down the production of galactaric acid from pectin using Trichoderma reesei', *Microbial Cell Factories*. BioMed Central, pp. 1–11.

Pohl, C., Kiel, J. A. K. W., Driessen, A. J. M., Bovenberg, R. A. L. and Nygård, Y. (2016) 'CRISPR/Cas9 Based Genome Editing of Penicillium chrysogenum', *ACS Synthetic Biology*, 5(7), pp. 754–764.

Ramachandran, S., Fontanille, P., Pandey, A. and Larroche, C. (2006) 'Gluconic Acid : Properties , Applications and Microbial Production', *Food Technology and Biotechnology*, 44(2), pp. 185–195.

Rautiainen, S., Lehtinen, P., Chen, J., Vehkamäki, M., Niemelä, K., Leskelä, M. and Repo, T. (2015) 'Selective oxidation of uronic acids into aldaric acids over gold catalyst', *RSC Advances*, 5(25), pp. 19502–19507.

Richard, P. and Hilditch, S. (2009) 'D-Galacturonic acid catabolism in microorganisms and its biotechnological relevance', *Applied Microbiology and Biotechnology*, 82, pp. 597–604.

Roukas, T. (2000) 'Citric and gluconic acid production from fig by Aspergillus niger using solid-state fermentation.', *Journal of industrial microbiology & biotechnology*, 25(6), pp. 298–304.

Sander, J. D. and Joung, J. K. (2014) 'CRISPR-Cas systems for editing, regulating and targeting genomes', *Nature Biotechnology*, 32(4), pp. 347–350.

Sarkari, P., Marx, H., Blumhoff, M. L., Mattanovich, D., Sauer, M. and Steiger, M. G. (2017) 'An efficient tool for metabolic pathway construction and gene integration for Aspergillus niger', *Bioresource Technology*. Elsevier Ltd, 245, pp. 1327–1333.

Sauermann, G. (2004) 'Cosmetic and dermatological preparations for the treatment of and active prevention of dry skin and or other negative alterations in the physiological homeostasis of healthy skin'.

Schuster, E., Dunn-Coleman, N., Frisvad, J. and Van Dijck, P. (2002) 'On the safety of Aspergillus niger - A review', *Applied Microbiology and Biotechnology*, 59(4–5), pp.

426–435.

Taguchi, Y., Oishi, A. and Iida, H. (2008) ‘One-step Synthesis of Dibutyl Furandicarboxylates from Galactaric Acid’, *Chemistry Letters*, 37(1), pp. 50–51.

Thompson, D. B., Aboulhouda, S., Hysolli, E., Smith, C. J., Wang, S., Castanon, O. and Church, G. M. (2017) ‘The future of multiplexed eukaryotic genome engineering’, *ACS Chemical Biology*, 13(2), pp. 313–325.

Wakai, S., Arazoe, T., Ogino, C. and Kondo, A. (2017) ‘Future insights in fungal metabolic engineering’, *Bioresource Technology*. Elsevier Ltd, 245, pp. 1314–1326.

Wang, J. (2016) *Characterization and engineering of hexaric and hexacuronic acid pathways in fungal microorganisms*.

Yelton, M. M., Hamer, J. E. and Timberlake, W. E. (1984) ‘Transformation of Aspergillus nidulans by using a trpC plasmid’, *Proceedings of the National Academy of Sciences of the United states of America*, 81(March), pp. 1470–1474.

Zheng, Y. M., Lin, F. L., Gao, H., Zou, G., Zhang, J. W., Wang, G. Q., Chen, G. D., Zhou, Z. H., Yao, X. S. and Hu, D. (2017) ‘Development of a versatile and conventional technique for gene disruption in filamentous fungi based on CRISPR-Cas9 technology’, *Scientific Reports*. Springer US, 7(1), pp. 1–10.

## APPENDIX A: PRIMER LIST

| Name | Sequence | Target |
|---|---|---|
| oPEEL-195 | AACGCCAGCAACGCGGCCTT | Common forward primer for entry vector sequencing |
| oPEEL-196 | TGGACTCCTGTTGATAGATC | Common reverse primer for entry vector sequencing |
| oPEEL-257 | GCATCGTCTCATCGGTCTCACAATATCGACAGCGAGTGAGATAT | Upstream 1000 Flank Forward Primer *gaaX* |
| oPEEL-258 | GCATCGTCTCATCGGTCTCACAATTCAAGATCCTCTCTTCCTTG | Upstream 500 Flank Forward Primer *gaaX* |
| oPEEL-259 | GCATCGTCTCATCGGTCTCACAATGATCTCTTCCTCCGCGATCG | Upstream 100 Flank Forward Primer *gaaX* |
| oPEEL-260 | ATGCCGTCTCAGGTCTCAAGGGCAGTATACTGCTCGTTCTCA | Upstream Flank Reverse Primer *gaaX* |
| oPEEL-261 | GCATCGTCTCATCGGTCTCAGAGTTGAACTGTATCCACTCCTGG | Downstream Flank Forward Primer *gaaX* |
| oPEEL-262 | ATGCCGTCTCAGGTCTCATCGGCCAGATTCTTGCAGGCGTTG | Downstream 1500 Flank Reverse Primer *gaaX* |
| oPEEL-263 | ATGCCGTCTCAGGTCTCATCGGTTGCAGGCCTCACAGCTG | Downstream 1000 Flank Reverse Primer *gaaX* |
| oPEEL-264 | ATGCCGTCTCAGGTCTCATCGGTGCCAGTCAGTAGTCGTCGT | Downstream 500 Flank Reverse Primer *gaaX* |
| oPEEL-265 | ATGCCGTCTCAGGTCTCATCGGCTGTTGGATCATCACACTGC | Downstream 100 Flank Reverse Primer *gaaX* |

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|---|---|---|
| oPEEL-266 | GCATCGTCTCATCGGTCTCACAATCGAGTGCCTTTCTGGACTAT | Correct Upstream 1500 Flank Forward Primer *gaaX* |
| oPEEL-267 | TGCCAGTGCTAGTTCCTTCG | *A. oryzae* test sequencing forward primer |
| oPEEL-268 | ATACCACTGACTCCGGTTTT | *A. oryzae* test sequencing forward DNA probe |
| oPEEL-271 | CATACTGAGCTGCCAATCAT | *A. oryzae* test sequencing forward DNA probe |
| oPEEL-272 | AAAGGAGGGCTGAGTGATCA | *A. oryzae* test sequencing reverse DNA probe |
| oPEEL-275 | GAATGTGTGCGATGGAGGTG | *A. oryzae* test sequencing reverse DNA probe |
| oPEEL-286 | GCATCGTCTCATCGGTCTCACAATTGACGCCTACTCTACAGCAC | Upstream 1500 Flank Forward Primer *gaaX Oryzae* |
| oPEEL-287 | ATGCCGTCTCAGGTCTCAAGGGACCAGGGTCGTGAATGAATG | Upstream 1500 Flank Reverse Primer *gaaX Oryzae* |
| oPEEL-290 | GCATCGTCTCATCGGTCTCATACACAGGCTTGTCAGATATGTTC | *pyrG* forward primer *A. oryzae* |
| oPEEL-291 | ATGCCGTCTCAGGTCTCAACTCAAATGGTGGCTAGGCTCTGA | *pyrG* Reverse Primer *A. oryzae* |
| oPEEL-292 | GAACATATCTGACAAGCCTG | *gaaX* deletion checking reverse primer in *pyrG* promoter |
| oPEEL-293 | TCAGAGCCTAGCCACCATTT | *gaaX* deletion checking forward primer in *pyrG* terminator |
| oPEEL-294 | GTTCTGGTCAACACGCATTG | *GaaX* check, forward in *gaaX* |
| oPEEL-295 | CATGTTGACCGCTGAGAGGA | *GaaX* check, reverse in *gaaX* |
| oPEEL-296 | GCATCGTCTCATCGGTCTCATATGTTGAGCTCCACCATGGCCT | udh to moclo type 3, forward |

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|---|---|---|
| oPEEL-297 | ATGCCGTCTCAGGTCTCAGGATGGTTACTTATCGCCGAAGGG | udh to moclo type 3, reverse |
| oPEEL-299 | ATGCCGTCTCAGGTCTCACATATTGATGTCTGCTCAAGCGGG | gpdA to moclo type 2, reverse |
| oPEEL-311 | GCATCGTCTCATCGGTCTCACAATACAGCATCCAAGACTCACAT | Upstream 1500 Flank Forward Primer *39114* niger |
| oPEEL-312 | ATGCCGTCTCAGGTCTCAAGG*GAAA*CGCCCTACTATATCGGA | Upstream 1500 Flank Reverse Primer *39114* niger |
| oPEEL-313 | GCATCGTCTCATCGGTCTCAGAGTGTAGGATTGAACAAGAGTGC | Downstream 1300 Flank Forward Primer *39114* niger |
| oPEEL-314 | ATGCCGTCTCAGGTCTCATCGGTTGGAGTC*GAAA*CCGTCCTC | Downstream 1300 Flank Reverse Primer *39114* niger |
| oPEEL-315 | GCATCGTCTCATCGGTCTCACAATCAATGTCAGCTATCCTCGTA | Upstream 1500 Flank Forward Primer *39114 oryzae* |
| oPEEL-316 | ATGCCGTCTCAGGTCTCAAGGGGGATAGGCTTCATTTTGGTG | Upstream 1500 Flank Reverse Primer *39114 oryzae* |
| oPEEL-317 | GCATCGTCTCATCGGTCTCAGAGTCTAACTCGATTAAGGTTGCC | Downstream 1300 Flank Forward Primer *39114 oryzae* |
| oPEEL-318 | ATGCCGTCTCAGGTCTCATCGGGCATGCAGTGAGTCAGAATA | Downstream 1300 Flank Reverse Primer *39114 oryzae* |
| oPEEL-320 | GCATCGTCTCATCGGTCTCAGAGTGATGAATCGGACTTATGTGC | Downstream 1171 Flank Forward Primer *gaaX oryzae*, without BsmBI site |
| oPEEL-321 | ATGCCGTCTCAGGTCTCATCGGGCCTCCAAGACATCTGTTTT | Downstream 1171 Flank Reverse Primer *gaaX oryzae*, without BsmBI site |
| oPEEL-322 | GCATCGTCTCATCGGTCTCAAACGTCAAGTGGGAGTTTGTGGAC | *gaaB* promoter Forward MoClo Type 2 |
| oPEEL-323 | ATGCCGTCTCAGGTCTCACATACAATTGAGAAGAACGACGAC | *gaaB* promoter Reverse MoClo Type 2 |
| oPEEL-324 | ATGCCGTCTCAGGTCTCACATAGGTGTCGGTTGTTTCTGTTC | Correct *gaaB* promoter Reverse MoClo Type 2 |

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|---|---|---|
| oPEEL-325 | GCATCGTCTCATCGGTCTCACAATGCTATCGAGTTTATCACGGC | Upstream 1500 flank forward real *gaaA* niger |
| oPEEL-326 | ATGCCGTCTCAGGTCTCAAGGGAGCCAGCGAACAATCCATTC | Upstream 1500 flank reverse real *gaaA* niger |
| oPEEL-327 | GCATCGTCTCATCGGTCTCAGAGTTAATTGACAGGGGCGAACAA | Downstream 1500 flank forward real *gaaA* niger |
| oPEEL-328 | ATGCCGTCTCAGGTCTCATCGGGGATCGAGGTAGGAGAACAC | Downstream 1500 flank reverse real *gaaA* niger |
| oPEEL-329 | GCATCGTCTCATCGGTCTCACAATCTTGGAGGGACAATGAGAGC | Upstream flank forward real *gaaA oryzae* |
| oPEEL-330 | ATGCCGTCTCAGGTCTCAAGGGTTGTGATCTGCTTTGGAGGA | Upstream flank reverse real *gaaA oryzae* |
| oPEEL-331 | GCATCGTCTCATCGGTCTCAGAGTAGGCTCATTAGGGTCGATCT | Downstream forward real *gaaA oryzae* |
| oPEEL-332 | ATGCCGTCTCAGGTCTCATCGGGGTGATGATGTGGTAGCTCC | Downstream reverse real *gaaA oryzae* |
| oPEEL-333 | CCACAATCTTCTTCTCGACG | *gaaB* checking protomoter |
| oPEEL-334 | GGC*GAAA*AAGGTATGGCAGA | new Δ*gaaX* downstream check for *A. niger* |
| oPEEL-335 | CCTCACCACCGATCTCATTG | Δ*gaaA* upstream check for *A. niger* |
| oPEEL-336 | GCTAGGGTGTTGTCCGTAAG | Δ*gaaA* downstream check for *A. niger* |
| oPEEL-337 | GACCTCCGACAA*GAAA*GTGG | *gaaA* check for *A. niger* forward |
| oPEEL-338 | TCTTGGGCTGACTCATGTAG | *gaaA* check for *A. niger* reverse |
| oPEEL-339 | GACCAGAGTTGACGACGATA | Δ*39114* upstream check for *A. niger* |

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|---|---|---|
| oPEEL-340 | GGAATCCTCATCCTCATCGT | Δ*39114* downstream check for *A. niger* |
| oPEEL-341 | TCGGACAGCTCTCAAAGAAC | *39114* check for *A. niger* forward |
| oPEEL-342 | AAGAGTTCTGTGCGAGTTGG | *39114* check for *A. niger* reverse |
| oPEEL-343 | TGTGTCGAGCTAACCCTAGA | Δ*gaaA* upstream check for A.*oryzae* |
| oPEEL-344 | ATATCTCCTCCCAGCTCGAC | Δ*gaaA* downstream check for A.*oryzae* |
| oPEEL-345 | GCTCCCACCTCCAAAAGAAT | *gaaA* check for *A. oryzae* forward |
| oPEEL-346 | AGAAGCAGTATAAACGCCCG | *gaaA* check for *A. oryzae* reverse |
| oPEEL-347 | GGTCTCACATTCCACGGTAA | Δ*39114* upstream check for *A. oryzae* |
| oPEEL-348 | ACGGATTCATTTCGCTCCTC | Δ*39114* downstream check for *A. oryzae* |
| oPEEL-349 | GCGGTTATGTTCGGAGGATC | *39114* check for *A. oryzae* forward |
| oPEEL-350 | GTTTCGGATAGGACAAGCGA | *39114* check for *A. oryzae* reverse |

# APPENDIX B: PLASMIDS

| Entry vectors | Description | Marker | Origin |
|---|---|---|---|
| pytk001 | Part Plasmid Entry Vector | CamR | (Lee *et al.* 2015) |
| pytk002 | ConLS | CamR | (Lee *et al.* 2015) |
| pytk048 | 2,3,4 type spacer | CamR | (Lee *et al.* 2015) |
| pytk051 | tENO1 | CamR | (Lee *et al.* 2015) |
| pytk067 | ConR1 | CamR | (Lee *et al.* 2015) |
| pytk089 | AmpR-ColE1 | AmpR | (Lee *et al.* 2015) |
| B6042 *UDH* | Bacterial uronate dehydrogenase gene | CamR | VTT strain collection |
| pytk001-*UDH* | Bacterial uronate dehydrogenase gene | CamR | This study |
| pytk001-*pyrG*o | *pyrG* from *A. oryzae* | CamR | This study |
| pytk001-1500U*gaaX*n | 1500bp upstream of *gaaX* in *A. niger* | CamR | This study |
| pytk001-1500D*gaaX*n | 1500bp downstream of *gaaX* in *A. niger* | CamR | This study |
| pytk001-1000U*gaaX*n | 1000bp upstream of *gaaX* in *A. niger* | CamR | This study |
| pytk001-1000D*gaaX*n | 1000bp downstream of *gaaX* in *A. niger* | CamR | This study |
| pytk001-500U*gaaX*n | 500bp upstream of *gaaX* in *A. niger* | CamR | This study |
| pytk001-500D*gaaX*n | 500bp downstream of *gaaX* in *A. niger* | CamR | This study |
| pytk001-100U*gaaX*n | 100bp upstream of *gaaX* in *A. niger* | CamR | This study |
| pytk001-100D*gaaX*n | 100bp downstream of *gaaX* in *A. niger* | CamR | This study |
| pytk001-1500U*gaaX*o | 1500bp upstream of *gaaX* in *A. oryzae* | CamR | This study |
| pytk001-1168D*gaaX*o | 1168bp downstream of *gaaX* in *A. oryzae* | CamR | This study |
| pytk001-1500U*39114*n | 1500bp upstream of *39114* in *A. niger* | CamR | This study |
| pytk001-1300D*39114*n | 1300bp downstream of *39114* in *A. niger* | CamR | This study |
| pytk001-1500U*39114*o | 1500bp upstream of *39114* in *A. oryzae* | CamR | This study |
| pytk001-1282D*39114*o | 1282bp downstream of *39114* in *A. oryzae* | CamR | This study |

| | | | |
|---|---|---|---|
| pytk001-1470U*gaaA*n | 1470bp upstream of *gaaA* in *A. niger* | CamR | This study |
| pytk001-1500D*gaaA*n | 1500bp downstream of *gaaA* in *A. niger* | CamR | This study |
| pytk001-1268U*gaaA*o | 1168bp upstream of *gaaA* in *A. oryzae* | CamR | This study |
| pytk001-1497D*gaaA*o | 1497bp downstream of *gaaA* in *A. oryzae* | CamR | This study |

| **Donor DNA cassettes** | **Description** | **Marker** | **Origin** | **Components** |
|---|---|---|---|---|
| 1500*gaaX*nDC | Donor DNA for the replacement of *gaaX* with *pyrG* in *A. niger* | AmpR | This study | pytk001-1500U*gaaX*n, pytk001-1500D*gaaX*n, pytk001-*pyrG*o, pytk-002,pytk-048,pytk-067,pytk-089 |
| 1000*gaaX*nDC | Donor DNA for the replacement of *gaaX* with *pyrG* in *A. niger* | AmpR | This study | pytk001-1000U*gaaX*n, pytk001-1000D*gaaX*n, pytk001-*pyrG*o, pytk-002,pytk-048,pytk-067,pytk-089 |
| 500*gaaX*nDC | Donor DNA for the replacement of *gaaX* with *pyrG* in *A. niger* | AmpR | This study | pytk001-500U*gaaX*n, pytk001-500D*gaaX*n, pytk001-*pyrG*o, pytk-002,pytk-048,pytk-067,pytk-089 |
| 100*gaaX*nDC | Donor DNA for the replacement of *gaaX* with *pyrG* in *A. niger* | AmpR | This study | pytk001-100U*gaaX*n, pytk001-100D*gaaX*n, pytk001-*pyrG*o, pytk-002,pytk-048,pytk-067,pytk-089 |
| *gaaX*nDC | Donor DNA for the replacement of *gaaX* with *UDH* and *pyrG* in *A. niger* | AmpR | This study | pytk001-1500U*gaaX*n, pytk001-1500D*gaaX*n, pytk001-*pyrG*o, pytk001-UDH, pytk-002,pytk-048,pytk-067,pytk-089 |
| *gaaX*oDC | Donor DNA for the replacement of *gaaX* with *UDH* and *pyrG* in *A. oryzae* | AmpR | This study | pytk001-1500U*gaaX*o, pytk001-1168D*gaaX*o, pytk001-*pyrG*o, pytk001-UDH, pytk-002,pytk-048,pytk-067,pytk-089 |

| | | | | |
|---|---|---|---|---|
| *gaaA*nDC | Donor DNA for the replacement of *gaaA* with *UDH* and *pyrG* in *A. niger* | AmpR | This study | pytk001-1470U*gaaA*n, pytk001-1500D*gaaA*n, pytk001-*pyrG*o, pytk001-UDH, pytk-002,pytk-048,pytk-067,pytk-089 |
| *gaaA*oDC | Donor DNA for the replacement of *gaaA* with *UDH* and *pyrG* in *A. oryzae* | AmpR | This study | pytk001-1497D*gaaA*o,pytk001-1268U*gaaA*o, pytk001-*pyrG*o, pytk001-UDH, pytk-002,pytk-048,pytk-067,pytk-089 |
| *39114*AnDC | Donor DNA for the replacement of *39114* with *UDH* and *pyrG* in *A. niger* | AmpR | This study | pytk001-1500U*39114*n, pytk001-1500D*39114*n, pytk001-*pyrG*o, pytk001-UDH, pytk-002,pytk-048,pytk-067,pytk-089 |
| *39114*AoDC | Donor DNA for the replacement of *39114* with *UDH* and *pyrG* in *A. oryzae* | AmpR | This study | pytk001-1500U*39114*o, pytk001-1282D*39114*o, pytk001-*pyrG*o, pytk001-UDH, pytk-002,pytk-048,pytk-067,pytk-089 |

# APPENDIX C:GRNA LIST

| Name | Protospacer in crRNA | Description |
|---|---|---|
| gERA-001 | GTGGAAGTGGACGTCGCCAG | *A. niger gaaX* version 1 |
| gERA-002 | GGTGATTGGCGGGTGAACGG | *A. niger gaaX* version 2 |
| gERA-009 | GCGTAAACCTCTTCAATCAG | *A. niger 39114* version 1 |
| gERA-010 | GGCTTCTCCGGACTGCACAG | *A. niger 39114* version 2 |
| gERA-015 | CTGAGTACGAAGG*GAAA*CGA | *A. niger gaaA* version 1 |
| gERA-016 | AAACTGACCGAGA*GAAA*CCA | *A. niger gaaA* version 2 |